

Polysorbate-Degrading Host Cell Proteins in Biopharmaceuticals

Identification, Characterization and Mechanistic Insights

Zur Erlangung des akademischen Grades einer
DOKTORIN DER NATURWISSENSCHAFTEN (DR. RER. NAT.)

von der KIT-Fakultät für Chemieingenieurwesen und Verfahrenstechnik des
Karlsruher Instituts für Technologie (KIT)
genehmigte

DISSERTATION

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Tag der mündlichen Prüfung: 20.07.2026

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Danksagung

Diese Dissertation entstand im Rahmen einer Direktkooperation zwischen dem Karlsruher Institut für Technologie (KIT) und dem Unternehmen Boehringer Ingelheim. An erster Stelle möchte ich mich ganz herzlich bei Prof. Dr. Matthias Franzreb bedanken, der mir die Möglichkeit gegeben hat, diese Arbeit anzufertigen. Vielen Dank für die hervorragende Betreuung und Unterstützung in den vergangenen Jahren, sowie die große wissenschaftliche Freiheit, die mir während dieser Zeit entgegengebracht wurde. Außerdem möchte ich mich bei Prof. Dr. Sonja Berensmeier herzlich für die Übernahme des Korreferats bedanken.

Ein großes Dankeschön gilt meinen talentierten Studenten: Lukas Griesinger, Stefan Schneider und Dominik Nitz. Die Zusammenarbeit mit euch war mir eine große Freude. Vielen Dank für eure Ideen, eure Motivation und eure großartige Unterstützung. Ohne euch hätte ich vieles nicht umsetzen können.

Mein besonderer Dank gilt Joey Studts für die kontinuierliche Betreuung und Unterstützung während meiner gesamten Zeit bei Boehringer Ingelheim, sowohl in wissenschaftlicher als auch in menschlicher Hinsicht. Darüber hinaus möchte ich mich bei Daniel Lakatos und Simon Kluters für ihre Unterstützung bedanken.

Ebenso danke ich allen Kollaborationspartnern bei Boehringer Ingelheim für die tolle Zusammenarbeit, insbesondere Linus Weiss, Viktor Gross, Simon Fischer und Patrick Gariel. Ohne euch wäre diese Arbeit in dieser Form nicht möglich gewesen.

Ich danke außerdem allen Kollegen der AG Franzreb für die gemeinsame Zeit, den fachlichen wie persönlichen Austausch bei Seminarausfahrten, Besuchen in Karlsruhe und Konferenzen und für die herzliche Aufnahme in der Arbeitsgruppe.

Meinen Dank gilt ebenso allen Kollegen in der Downstream Entwicklung, die mich stets mit technischem Support im Labor unterstützt haben. Danke für die freundschaftliche und produktive Arbeitsatmosphäre auf unserem Stockwerk. Ich möchte mich auch bei meinem neuen Labor und dem Downstream Modeling Team für die herzliche Aufnahme bedanken. Ich freue mich auf die kommende gemeinsame Zeit.

Zuletzt danke ich meiner Familie, insbesondere meinen Eltern, meinem Bruder Manuel und meinem Partner Julian, für die stetige Unterstützung, den Rückhalt und das Verständnis während dieser Zeit.

Zusammenfassung

Polysorbate sind weit verbreitete Hilfsstoffe in biopharmazeutischen Formulierungen, insbesondere in monoklonalen Antikörperpräparaten. Sie übernehmen eine zentrale Funktion bei der Stabilisierung von Proteinen, indem sie Aggregation, Oberflächenadsorption und Grenzflächenstress reduzieren. Aufgrund ihrer amphiphilen Eigenschaften schützen Polysorbate therapeutische Proteine während Herstellung, Lagerung und Applikation und sind daher in der Mehrzahl zugelassener Antikörperformulierungen enthalten. Trotz ihrer langjährigen Anwendung und regulatorischen Akzeptanz sind Polysorbate jedoch chemisch und enzymatisch instabil. Der Abbau von Polysorbat hat sich als wesentliche Ursache für Formulierungsinstabilitäten erwiesen und stellt eine der größten Herausforderungen während der biopharmazeutischen Entwicklung dar.

In den vergangenen Jahren wurde die enzymatische Hydrolyse durch residuale Wirtszellproteine als vorherrschender Mechanismus des Polysorbatabbaus identifiziert. Bereits sehr geringe Mengen katalytisch aktiver Hydrolasen, die im Endprodukt vorliegen, können Esterbindungen innerhalb der Polysorbate spalten. Dieser Prozess führt zur Freisetzung freier Fettsäuren, einem fortschreitenden Verlust der Tensidfunktion sowie zur Bildung von Partikeln. Diese Abbauprodukte können die Produktstabilität beeinträchtigen, die Haltbarkeit reduzieren und potenzielle Sicherheitsrisiken darstellen. Die Relevanz dieses Problems wird durch zahlreiche berichtete Fälle von Partikelbildung in Polysorbat haltigen Formulierungen unterstrichen, in denen enzymatischer Abbau als Ursache identifiziert werden konnte.

Aktuelle Entwicklungen in der biopharmazeutischen Industrie erhöhen zusätzlich die Anfälligkeit von Formulierungen gegenüber enzymatischem Polysorbatabbau. Intensivierte Upstream-Prozesse führen zu höheren Zellkulturtitern und damit zu einer erhöhten absoluten Menge an Wirtszellproteinen, die in die Downstream-Aufreinigung gelangen. Gleichzeitig verschiebt sich die therapeutische Landschaft hin zu komplexeren Molekülformaten wie multispezifischen Antikörpern, Antikörper-Wirkstoff-Konjugaten oder Fc-Fusionsproteinen. Diese Formate stellen etablierte Reinigungsstrategien vor neue Herausforderungen und können die Abreicherung von Wirtszellproteinen verändern. Hinzu kommt der Trend zu hochkonzentrierten Formulierungen für die subkutane Applikation, bei denen residuale Verunreinigungen während Ultrafiltration und Diafiltration mitkonzentriert werden. Zusammengenommen erhöhen diese Faktoren die Wahrscheinlichkeit, dass enzymatisch

aktive Hydrolasen im Endprodukt verbleiben und während der Lagerung Polysorbate abbauen.

Obwohl der Abbau von Polysorbat intensiv untersucht wurde, bleiben mehrere zentrale Fragen ungelöst. Standardmethoden zur Bestimmung von Wirtszellproteinen, etwa generische Enzym-gekoppelte Immunadsorptionsassays (im Englischen „Enzyme-Linked Immunosorbent Assay“, „ELISA“), erfassen lediglich die Gesamtverunreinigungen, liefern jedoch keine Informationen über einzelne Proteine oder deren funktionelle Relevanz. Massenspektrometrische Verfahren ermöglichen zwar die Identifikation spezifischer Wirtszellproteine, stoßen jedoch in formulierten Produkten mit hohen Antikörperkonzentrationen häufig an Sensitivitätsgrenzen. Zudem bedeutet der Nachweis eines Proteins nicht zwangsläufig, dass dieses unter Formulierungsbedingungen enzymatisch aktiv gegenüber Polysorbat ist. Folglich bleibt in vielen Fällen unklar, welche Hydrolasen tatsächlich für den Polysorbatabbau verantwortlich sind und weshalb bestimmte Enzyme den Downstream Prozess bestehen, während andere effektiv entfernt werden.

Ziel der vorliegenden publikationsbasierten Dissertation war es daher, polysorbat-abbauende Wirtszellhydrolasen aus komplementären analytischen, biochemischen und prozess-technischen Perspektiven zu untersuchen und ein integriertes Verständnis ihrer Identität, Persistenz und funktionellen Relevanz entlang der biopharmazeutischen Herstellungskette zu entwickeln. Zur Bearbeitung dieser Fragestellung wurden fünf aufeinander aufbauende Studien durchgeführt, die jeweils unterschiedliche Aspekte der Polysorbat-Degradation adressieren und gemeinsam ein umfassendes Gesamtbild ergeben.

Die erste Publikation (Kapitel 3) befasst sich mit der Identifikation und funktionellen Charakterisierung polysorbat-abbauender Hydrolasen aus Expressionssystemen der Ovarialzellen des Chinesischen Hamsters (im Englischen „Chinese hamster ovary“, „CHO“). Mittels sensitiver massenspektrometrischer Analysen formulierter monoklonaler Antikörperprodukte wurde ein definiertes Set potenzieller Ester-spaltender Hydrolasen identifiziert. Für die funktionelle Charakterisierung wurde eine Lipoproteinlipase-Knockout-Produktionszelllinie etabliert, um einen dominanten Beitrag zur Polysorbat-Degradation auszuschalten. Die rekombinante Expression und orthogonale Aufreinigung der verbleibenden Kandidaten ermöglichte eine systematische Untersuchung ihrer enzymatischen Aktivität unter formulierungsspezifischen Bedingungen. Die Ergebnisse zeigen, dass die Polysorbate-Degradation nicht durch ein einzelnes Enzym verursacht wird, sondern durch eine definierte Gruppe von Hydrolasen mit unterschiedlicher zellulärer Lokalisation, Sekretionscharakteristik und Abbaukinetik. Unter den aktiven Hydrolasen zeigte sich konsistent eine konservierte Serin-Histidin-Aspartat/Glutamat-Triade, während nicht-aktive Enzyme dieses Motiv nicht aufwiesen.

Die zweite Publikation (Kapitel 4) untersucht das Verhalten polysorbat-abbauender Hydrolasen während der Downstream-Aufreinigung. Mithilfe von Hochdurchsatz-Bindungs-screens, einer durch statistische Versuchsplanung gestützten Optimierung an Miniatursäulen sowie chromatographischen Validierungsexperimenten wurde das Verhalten einzelner Hydrolasen im Vergleich zu unterschiedlichen Antikörperformaten während Kationenaustausch und multimodalen chromatographischen Reinigungsschritten systematisch analysiert. Die Ergebnisse zeigen, dass eine durch ähnliche physikochemische Eigenschaften bedingte Co-Elution einen wesentlichen Mechanismus für die Persistenz von Hydrolasen darstellt. Zudem hängt das Ausmaß der Entfernung stark von den Eigenschaften des jeweiligen Antikörpers ab, was erklärt, weshalb Plattformprozesse keine universelle Abtrennung von polysorbat-abbauenden Enzymen für unterschiedliche Moleküle gewährleisten können.

Die dritte Publikation (Kapitel 5) erweitert diese Erkenntnisse um die Untersuchung direkter Interaktionen zwischen Wirtszellproteinen und Antikörpern als zusätzlichen Persistenzmechanismus. Hierzu wurde ein auf Biolayer-Interferometrie basierender Assay entwickelt, um transiente Interaktionen zwischen polysorbat-abbauenden Hydrolasen und verschiedenen monoklonalen Antikörpern zu quantifizieren. Die gemessenen Wechselwirkungen zeigten schnelle Assoziations- und Dissoziationskinetiken mit Gleichgewichtsdissoziationskonstanten im niedrigen nanomolaren Bereich. Trotz ihrer Transienz können diese Interaktionen zu einem Mittransport der Hydrolasen während des Protein A Schritts führen, was im Englischen als „Hitchhiking“ bezeichnet wird. Validierungsexperimente mittels Protein A Chromatographie bestätigten, dass der verbleibende Hydrolasenanteil nach der Elution mit der Interaktionsstärke korreliert. Damit wird gezeigt, dass Hydrolasen nicht nur durch Co-Elution während Kationentausch- und multimodaler Chromatographie, sondern auch durch antikörpervermittelte Interaktionen während des Protein A Schrittes persistieren können.

Während die vorangegangenen Arbeiten Identität und Persistenz der Hydrolasen adressieren, widmet sich der letzte Teil der Dissertation ihrer funktionellen Aktivität gegenüber Polysorbat. Die vierte Publikation (Kapitel 6) untersucht die Enzymaktivität auf Ebene einzelner Polysorbat-Subspezies, die aus der heterogenen Zusammensetzung von Polysorbaten resultieren. Dabei zeigte sich, dass einzelne Hydrolasen unterschiedliche Substratpräferenzen hinsichtlich Mono-, Di-, und Multiester-Spezies, Kopfgruppenchemie und Fettsäurekettenlänge aufweisen. Die resultierenden Abbauprofile führen zu enzym-spezifischen Polysorbat-Schnittmustern (im Englischen „Fingerprints“). Der Vergleich mit etablierten indirekten Aktivitätsassays verdeutlicht, dass solche Methoden die enzymatische Aktivität in Abhängigkeit von der jeweiligen Substratspezifität nicht zuverlässig abbilden.

Die fünfte Publikation (Kapitel 7) greift das Konzept der enzym-spezifischen Polysorbat-Schnittmuster auf und etabliert einen Klassifizierungsansatz mithilfe von maschinellem Lernen (im Englischen „Machine Learning“) zur funktionellen Identifikation von Hydrolasen. Auf normalisierten Schnittmuster-Daten trainierte Klassifikationsmodelle erreichten eine hohe Vorhersagegenauigkeit und zeigten, dass enzym-spezifische Informationen unabhängig von Enzymkonzentration oder Degradationszeit im Abbauprofil erhalten bleiben. Eine externe Validierung bestätigte die Robustheit dieses Ansatzes und unterstreicht sein Potenzial, verantwortliche Hydrolasen auch dann zu identifizieren, wenn massenspektrometrische Nachweise limitiert sind oder wenn die primär verantwortliche Hydrolase nicht eindeutig identifiziert werden kann.

Zusammenfassend liefert diese Dissertation eine umfassende Untersuchung polysorbat-abbauender Wirtszellhydrolasen im Kontext der biopharmazeutischen Herstellung. Durch die Verknüpfung von Enzymidentifikation, Analyse von Persistenzmechanismen im Downstream, Interaktionsstudien sowie funktioneller Abbaucharakterisierung wird deutlich, dass die Polysorbat-Degradation ein multifaktorielles Problem darstellt, das durch eine definierte Gruppe von Hydrolasen verursacht wird. Die Ergebnisse tragen zu einem vertieften mechanistischen Verständnis bei und leisten einen substantziellen Beitrag zur Entwicklung von Strategien zur Risikominimierung, etwa durch gezielte Modifikation von Zelllinien, angepasste Downstream Prozesse und funktioneller Überwachung kritischer Wirtszellhydrolasen.

Abstract

Polysorbates are widely used excipients in biopharmaceutical formulations, particularly in monoclonal antibody drug products, where they play a critical role in stabilizing proteins against aggregation, surface adsorption, and interfacial stress. Due to their amphiphilic nature, polysorbates protect therapeutic proteins during manufacturing, storage, and administration. As a result, they are included in the vast majority of approved antibody formulations. Despite their long-standing use and broad regulatory acceptance, polysorbates are chemically and enzymatically labile. Their degradation has emerged as a major cause of formulation instability and has become a recurring challenge in biopharmaceutical development.

In recent years, enzymatic hydrolysis by residual host cell proteins has been identified as the predominant mechanism driving polysorbate degradation. Even trace levels of catalytically active hydrolases, often present at sub-ppm concentrations in the final drug product, can cleave ester bonds within polysorbates. This process leads to the release of free fatty acids, a gradual loss of surfactant functionality, and the formation of visible or subvisible particles. These degradation products can compromise product stability, reduce shelf life, and pose potential safety concerns. The industrial relevance of this problem is underscored by numerous reported cases of particle formation in polysorbate-containing formulations, where enzymatic degradation could be identified as the root cause.

Current trends in biopharmaceutical manufacturing have further increased the susceptibility of formulations to enzymatic polysorbate degradation. Intensified upstream processes lead to higher cell culture titers and consequently increase the absolute amount of host cell proteins entering downstream purification. At the same time, the therapeutic landscape is shifting toward increasingly complex molecular formats, such as multispecific antibodies, antibody-drug conjugates, and Fc-fusion proteins. These formats challenge established purification strategies and may alter host cell protein clearance behavior. In addition, the growing demand for high-concentration formulations to enable subcutaneous administration results in the co-concentration of residual impurities during ultrafiltration and diafiltration. Together, these developments increase the likelihood that enzymatically active hydrolases remain in the final product and degrade polysorbates during storage.

Although polysorbate degradation has been investigated extensively, several key questions remain unresolved. Standard host cell protein assays, such as generic Enzyme-Linked Immunosorbent Assays (ELISAs), measure total impurity levels but do not provide information on individual proteins or their functional relevance. Mass spectrometry can identify specific host cell proteins, but sensitivity is often limited in formulated drug products dominated by high antibody concentrations. Furthermore, detecting a protein does not necessarily mean that it is enzymatically active under formulation conditions. As a result, it is frequently unclear which hydrolases are responsible for polysorbate degradation in a given product and why certain enzymes persist through downstream processing while others are effectively removed.

The aim of this publication-based thesis was therefore to investigate polysorbate-degrading host cell hydrolases from complementary analytical, biochemical, and process-related perspectives and to develop a coherent understanding of their identity, persistence, and functional relevance throughout the biopharmaceutical manufacturing chain. To address this objective, five interconnected studies were conducted, each focusing on a specific aspect of polysorbate degradation while contributing to a comprehensive overall picture.

The first publication (Chapter 3) focuses on the identification and functional characterization of polysorbate-degrading hydrolases derived from Chinese hamster ovary expression systems. Using sensitive mass spectrometry-based analyses of formulated monoclonal antibody products, a defined set of candidate hydrolases acting on ester bonds was identified. To enable unbiased functional characterization, a lipoprotein lipase knockout production cell line was generated, eliminating a dominant contributor to polysorbate degradation. Recombinant expression and orthogonal purification steps of the remaining candidate hydrolases allowed a systematic assessment of their enzymatic activity under formulation-relevant conditions. These studies demonstrated that polysorbate degradation is not driven by a single enzyme but by a discrete group of hydrolases with distinct cellular localization, secretion behavior, and degradation kinetics. A conserved serine-histidine-aspartate/glutamate catalytic triad was observed among the hydrolases that were active against polysorbate in this study, whereas enzymes lacking activity did not contain this motif. This pattern suggests that the triad may be indicative of polysorbate-degrading activity, while not excluding the possibility of alternative catalytic mechanisms.

The second publication (Chapter 4) investigates the behavior of polysorbate-degrading hydrolases during downstream purification. Using high-throughput binding screenings, design-of-experiments-guided miniature column optimization, and chromatographic validation runs, the partitioning behavior of individual hydrolases was systematically compared with that of different monoclonal antibody formats during cation exchange and mixed-mode polishing steps. This study demonstrates that co-elution driven by similar

physicochemical properties represents a major mechanism underlying hydrolase persistence during polishing. Importantly, the extent of hydrolase removal was found to depend strongly on the properties of the antibody itself, explaining why standard purification processes cannot universally ensure clearance of polysorbate-degrading enzymes across different molecules.

In the third publication (Chapter 5), direct host cell protein-antibody interactions are investigated as an additional mechanism contributing to hydrolase persistence. A Biolayer interferometry-based assay was developed to quantify transient interactions between polysorbate-degrading hydrolases and different monoclonal antibodies. The measured interactions were characterized by fast association and dissociation kinetics, yielding apparent equilibrium dissociation constants in the low nanomolar range. Despite their transient nature, these interactions are sufficient to promote hydrolase carryover during Protein A capture, a phenomenon commonly referred to as hitchhiking. Validation experiments using protein A chromatography demonstrated that hydrolase retention correlated with interaction strength. This mechanism complements the findings of Chapter 4 by showing that, beyond co-elution during polishing, hydrolases can also persist during the Protein A capture step through antibody interactions.

While the preceding studies focused on hydrolase identity and persistence, the final part of this dissertation addressed their functional impact on polysorbates. The fourth publication (Chapter 6) focuses on the activity of polysorbate-degrading hydrolases against polysorbates at the subspecies level, which arise from the heterogeneous composition of polysorbates. These analyses revealed that individual hydrolases exhibit distinct substrate preferences for mono-, di-, and multiester species, different head group chemistries, and fatty acid chain lengths. The resulting degradation patterns lead to enzyme-specific polysorbate fingerprints. Comparison with commonly used indirect activity assays demonstrated that such methods can substantially misrepresent enzymatic activity depending on hydrolase specificity.

The fifth publication (Chapter 7) builds on the concept of enzyme-specific polysorbate fingerprints and introduces a supervised machine learning approach for functional hydrolase identification. Classification models trained on normalized fingerprint data achieved high predictive accuracy, demonstrating that enzyme-specific information is preserved in degradation patterns independent of enzyme concentration or degradation time. External validation confirmed the robustness of this approach and highlighted its potential to identify hydrolases responsible for polysorbate degradation even when mass spectrometry-based detection is limited or when the predominant enzymatic contributor is unknown.

In conclusion, this dissertation provides a comprehensive investigation of polysorbate-degrading host cell hydrolases in biopharmaceutical manufacturing. By integrating enzyme identification, downstream persistence mechanisms, interaction analysis, and functional degradation profiling, it demonstrates that polysorbate degradation is a multifactorial problem driven by a defined group of hydrolases whose relevance cannot be assessed by abundance alone. The results contribute to a deeper mechanistic understanding of polysorbate degradation and provide a substantial contribution to the development of risk-based mitigation strategies, including targeted cell line engineering, tailored downstream purification, and functional monitoring of critical host cell hydrolases.

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1 Introduction

The development and manufacture of biopharmaceuticals, and monoclonal antibodies in particular, have become one of the cornerstones of modern biomedicine. Over the past two decades, biologics have transformed the treatment landscape across oncology, immunology, and chronic inflammatory diseases [135].

In industrial monoclonal antibody production, Chinese hamster ovary (CHO) cells are the preferred host system due to their productivity and ability to generate human-compatible post-translational modifications. Yet, like all mammalian expression hosts, CHO cells secrete or release a diverse set of host cell proteins (HCPs) during cultivation [116,233]. Even though downstream purification processes efficiently remove the vast majority of these impurities, trace levels of HCPs inevitably remain. A subset of these HCPs has the potential to adversely affect product stability, potency, or safety [19,91,118]. A particular challenge is the stability of polysorbate-containing formulations, as trace amounts of host cell proteins can contribute to their degradation [48].

Polysorbates, most commonly PS20 and PS80, are currently regarded as indispensable excipients in monoclonal antibody formulations [221]. Nearly all commercial biologics include polysorbates to prevent aggregation and protect against interfacial stress [16,222]. Despite their long-standing use and critical role in stabilizing proteins, polysorbates are chemically instable. They can undergo oxidation and hydrolysis, leading to the formation of insoluble fatty acids and visible particles [48]. Enzymatic hydrolysis by residual host cell hydrolases is now widely recognized as the primary driver of polysorbate degradation. These enzymes, present at trace levels and often below the detection limit of conventional HCP assays, can cleave the ester bonds in polysorbates [118].

This challenge is amplified by several advances in biopharmaceutical manufacturing. First, upstream titers have increased dramatically in recent years [96]. This leads to an increased load of impurities on the downstream purification process [204]. Second, the biopharmaceutical landscape is shifting towards increasingly complex molecular formats, including multispecific antibodies and Fc-fusion proteins [135]. As a result, downstream selectivity becomes more challenging, and purification processes must be adapted [4,113,195]. Third, modern drug products frequently require high-concentration formulations (100-200 mg/mL) to enable subcutaneous administration [184,192]. Such formulations not only concentrate the therapeutic protein, but inevitably also co-concentrate any residual HCPs during ultrafiltration and diafiltration. This further raises the susceptibility to enzymatic polysorbate degradation.

These converging trends highlight the need for a mechanistic understanding of polysorbate-degrading hydrolases across the entire manufacturing chain. Polysorbate degradation represents a cross-functional challenge that spans cell line development, process engineering, and analytical characterization. Without a mechanistic understanding of the enzymes involved and their behavior throughout the manufacturing process, it becomes difficult to mitigate these critical host cell proteins.

This cumulative dissertation aims to address this gap by investigating polysorbate-degrading hydrolases from different complementary angles. Across five publications, the work examines the identification and differentiation of candidate hydrolases, their chromatographic behavior and persistence during downstream purification, their interactions with monoclonal antibodies, and how their functional degradation patterns across polysorbates can be used to assign degradation patterns to specific enzymes.

The following sections provide the scientific foundation for this dissertation. Section 1.1 introduces monoclonal antibodies and their structural characteristics relevant to manufacturing. Section 1.2 describes the role of polysorbates in therapeutic formulations and the mechanisms underlying their degradation. Section 1.3 discusses host cell proteins with a focus on hydrolases involved in polysorbate degradation. Section 1.4 introduces analytical tools for identifying and characterizing these enzymes, while Section 1.5 outlines mitigation approaches across cell line engineering, bioprocess optimization, and formulation design. Together, these sections present the conceptual foundation for the studies presented in this dissertation.

1.1 Monoclonal Antibodies

Monoclonal antibodies (mAbs) represent the most successful class of biopharmaceuticals. They offer targeted and effective treatments for a wide range of diseases, including cancer, infectious diseases, and autoimmune disorders [214,215]. Continuous advancements in antibody engineering and bioprocessing have led to the approval of more than 200 therapeutic products by 2025 [38].

Most therapeutic mAbs belong to the immunoglobulin G (IgG) class and are produced in mammalian expression systems. They combine a highly specific antigen-binding capability with the unique ability to mimic or enhance the body's natural immune response.

1.1.1 Structural and Functional Characteristics

An IgG antibody is a Y-shaped glycoprotein of approximately 150 kDa composed of two identical heavy (H) and two identical light (L) chains connected by disulfide bonds (Figure 1.1). Each chain has a variable region (V) and a constant region (C). The variable domains of one heavy and one light chain together form the antigen-binding fragment (Fab), while the two heavy-chain constant regions (CH2, CH3) form the crystallizable fragment (Fc), which mediates interaction with the immune system. The antigen-binding site is generated by six complementarity-determining regions (CDRs) located in the variable domains of both chains, which determine specificity and affinity toward a defined target epitope. The hinge region connects the Fab arms with the Fc portion and allows structural flexibility [33,55,178].

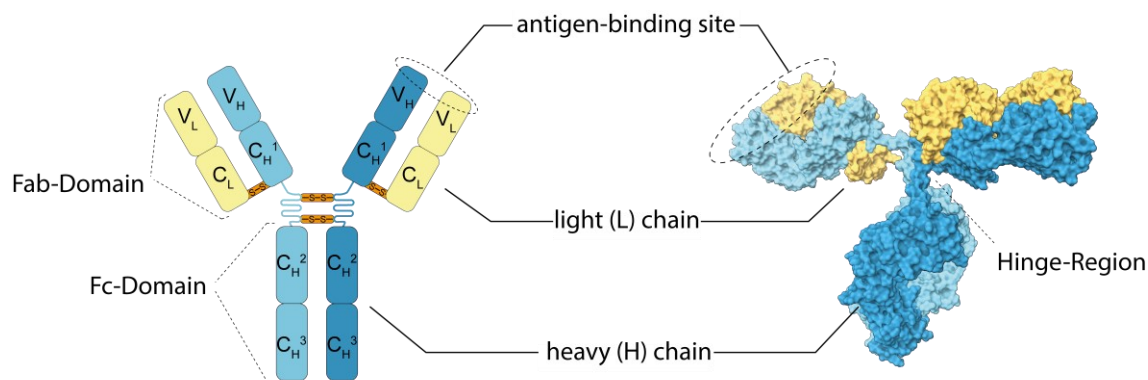


Figure 1.1: Structural organization of a human IgG antibody. The left schematic illustrates the Y-shaped architecture, which is composed of two heavy (H) and two light (L) chains. These chains form the antigen-binding Fab domains and the Fc domain, which is responsible for effector functions. The antigen-binding site is located in the variable regions (V_H and V_L), and the hinge region provides flexibility. The molecular surface representation on the right illustrates the three-dimensional organization of the antibody domains.

Human IgG antibodies are subdivided into four subclasses: IgG1, IgG2, IgG3, and IgG4. Among them, IgG1 and IgG4 are predominantly used in biopharmaceuticals [26,94]. All IgG subclasses contain a conserved glycosylation site within the Fc region. The

glycosylation pattern of an antibody is a key determinant of its pharmacokinetic and pharmacodynamic characteristics.

Over the past two decades, a broad range of engineered antibody formats has been developed to enhance the overall therapeutic potential of conventional IgG molecules [33]. Bispecific antibodies are designed to recognize two different antigens or epitopes simultaneously, enabling applications such as T-cell engagement [20]. Antibody-drug conjugates (ADCs) combine the specificity of antibodies with the cytotoxic potency of small molecules, allowing targeted drug delivery to malignant cells while minimizing systemic toxicity [223]. Fc-fusion proteins are engineered constructs that link the Fc region of an antibody to a functional protein domain, such as a receptor, ligand, or enzyme [123,163].

1.1.2 Manufacturing Process

The industrial production of monoclonal antibodies relies on mammalian expression systems, with Chinese hamster ovary (CHO) cells as the preferred host. CHO cells offer the advantage of robust growth in suspension culture and the capacity to perform complex human-like post-translational modifications [218]. A typical production process comprises cell line development and clone selection, an upstream phase, encompassing large-scale fed-batch or perfusion cultivation [116], and a downstream purification phase that isolates the antibody from impurities such as HCPs, DNA, endotoxins, and product-related impurities such as aggregates [11,164]. After purification, the antibody must be formulated with stabilizing excipients to ensure long-term stability, efficacy, and safety during storage and administration. A schematic overview of the manufacturing process is shown in Figure 1.2.

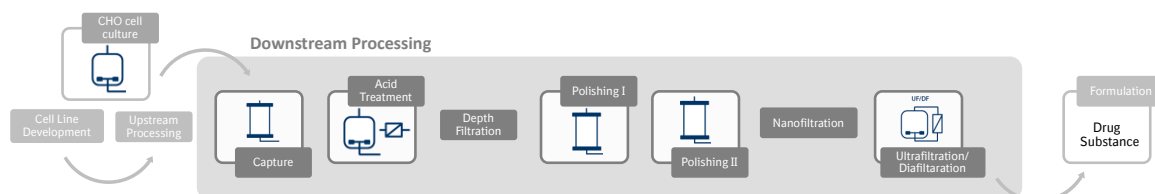


Figure 1.2: Schematic overview of the industrial monoclonal antibody manufacturing process. The workflow comprises cell line development and upstream processing in CHO cells followed by downstream purification steps including capture (Protein A chromatography), viral inactivation, depth filtration, polishing steps, nanofiltration, and ultrafiltration/diafiltration. The final stage involves formulation with stabilizing excipients to ensure product stability and safety.

Upstream development begins with stable transfection of the antibody gene cassette into CHO cells, followed by selection of high-producing clones. Process parameters including medium composition and feeding strategy influence not only productivity but also product quality attributes such as glycosylation profiles and charge variants [132]. During cell cultivation, thousands of host cell proteins are secreted or released from cells through lysis [152]. These host cell proteins are process-related impurities that must be effectively removed during downstream purification [19].

Downstream processing typically involves a sequence of chromatography and filtration steps, each designed to separate the monoclonal antibody based on its physicochemical properties from various impurities [95,122,137,186]. Protein A affinity chromatography serves as the primary capture step, exploiting the high-affinity interaction between the Fc region and the Protein A ligand [80]. The mAb is eluted with a low-pH buffer, which disrupts its binding to Protein A and enables collection of the purified antibody [81]. Following Protein A chromatography, the mAb solution undergoes low-pH viral inactivation, which is one of the two orthogonal steps required to remove viral contaminants [141]. Subsequent polishing steps, such as cation exchange (CEX), anion exchange (AEX), and mixed-mode chromatography (MMC), remove product-related variants (e.g. charge or size variants) and remaining process-related impurities such as host cell proteins [97,98,108]. Ion exchange and hydrophobic interactions are commonly used as separation principles. Anion exchange chromatography (AEX) binds negatively charged impurities like DNA to positively charged ligands. It is typically run in flow-through mode, where the mAb passes through while impurities are retained. In contrast, cation exchange chromatography (CEX) binds positively charged species and is operated in bind-and-elute mode to separate charge variants via salt gradients [191]. Mixed-mode chromatography (MMC) combines multiple interaction types, such as electrostatic and hydrophobic, in a single step, increasing selectivity and potentially replacing multiple polishing steps [234].

The final step in downstream processing is ultrafiltration and diafiltration (UF/DF), which concentrates the mAb and exchanges it into the formulation buffer [74,196]. A semi-permeable membrane retains the antibody while allowing smaller molecules like salts to pass through. Diafiltration against the formulation buffer then adjusts the final composition and concentration. Stabilizing excipients such as polysorbates are added during the formulation stage to protect the antibody against aggregation and surface adsorption during storage and administration [24].

The development of downstream processing steps aims to optimize product purity, process efficiency, and robustness while meeting regulatory standards for safety and efficacy. These requirements are defined by regulatory authorities such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) [3,111].

1.2 Polysorbates (PS)

Polysorbates are the most widely used excipients in biopharmaceutical formulations and have been employed for decades to enhance the stability of therapeutic proteins. Their amphiphilic nature allows them to reduce surface tension, prevent protein adsorption at interfaces, and mitigate aggregation or particle formation. Among non-ionic surfactants, polysorbate 20 (PS20) and polysorbate 80 (PS80) are most commonly used to formulate the final drug product [99,102]. Their amphiphilic structure enables them to form a protective layer at air-liquid or container-liquid interfaces. Consequently, the integrity of polysorbates during storage is directly linked to the overall stability and safety of the drug product. A comprehensive understanding of their chemical composition, functional

properties, and degradation pathways is therefore essential to ensure the long-term quality and efficacy of monoclonal antibody formulations [170].

1.2.1 Chemical Structure and Heterogeneity

Polysorbates are polyoxyethylene sorbitan fatty acid esters synthesized by ethoxylation and esterification of sorbitol and its anhydrides. PS20 and PS80 are produced by reacting sorbitan (a dehydrated form of sorbitol) with ethylene oxide to form polyoxyethylene (POE) chains, followed by partial esterification with predominantly lauric (C12:0) and oleic (C18:1) fatty acids, respectively [102,136]. The result is a highly heterogeneous mixture of molecules differing in the number of POE units, degree of esterification, and fatty acid composition. Typically, high-purity grade PS20 contains 40-60 % lauric acid, whereas high-purity grade PS80 contains >58 % oleic acid, along with minor amounts of other fatty acids such as myristic (C14:0), palmitic (C16:0), and stearic (C18:0) acids [211].

The structural diversity extends to the head group chemistry, which comprises sorbitan, isosorbide, and POE moieties. Each configuration yields different physicochemical properties, such as hydrophobicity, hydrodynamic radius, and critical micelle concentration [103]. As a result, a given polysorbate batch can contain hundreds of distinct subspecies, including mono-, di-, and multiesters. The relative proportion of these subspecies determines the surfactant's self-assembly behavior and its stabilizing performance [199]. Analytical deconvolution of this complexity is challenging, motivating the adoption of advanced analytical methods such as reverse-phase ultra-performance liquid chromatography coupled with mass spectrometry.

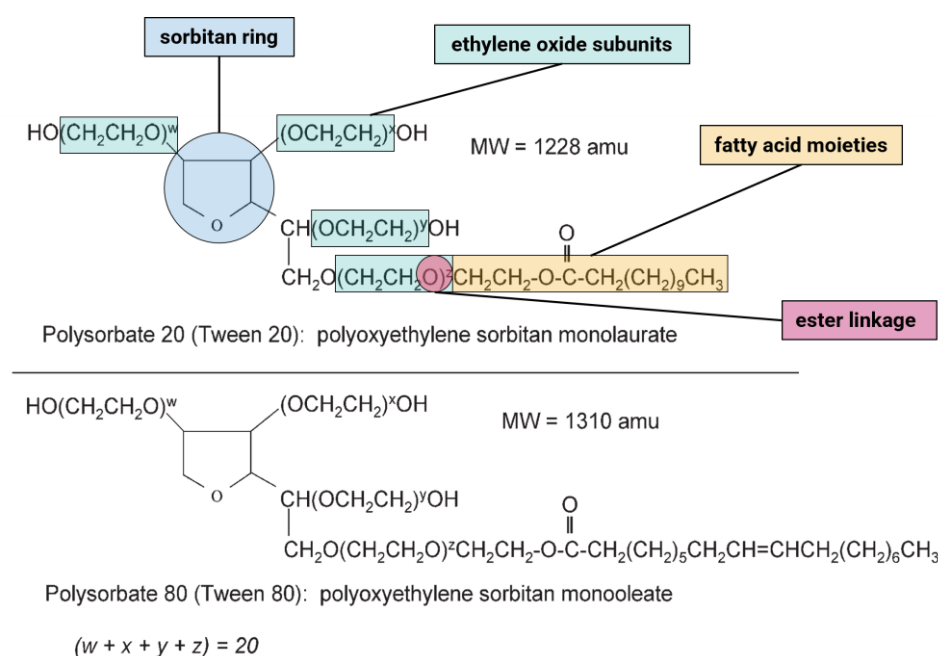


Figure 1.3: Chemical structure of polysorbate (PS) surfactants PS20 and PS80. Both share a sorbitan core that can exist as sorbitan or isosorbide, linked to polyoxyethylene (POE) chains. The notation $(w + x + y + z = 20)$ indicates the total number of ethylene oxide units distributed across four POE chains attached to the sorbitan ring. PS20 and PS80

differ primarily in their dominant fatty acid moieties: PS20 contains lauric acid (C12:0), whereas PS80 contains oleic acid (C18:1). However, depending on the polysorbate grade, additional fatty acids are present in varying proportions. Combined with variations in head group chemistry (sorbitan vs. isosorbide) and POE chain length, polysorbates are a highly complex and heterogeneous mixture of molecules. Figure modified from [99].

1.2.1.1 Functional Role in Protein Stabilization

The primary function of polysorbates in antibody formulations is to mitigate aggregation by shielding proteins from interfacial and mechanical stress. Proteins adsorbed at air-liquid or solid-liquid interfaces can unfold and form aggregates upon agitation, freeze-thaw cycling, or shear stress. Polysorbates competitively adsorb to these interfaces, forming a protective monolayer that prevents protein unfolding and aggregation. They also reduce the formation of interfacial foam and inhibit particle formation in vials [92,100].

From a thermodynamic perspective, polysorbates act as surface-active agents that lower the Gibbs free energy of interface formation. Their amphiphilic structure comprises a hydrophobic fatty acid tail and a hydrophilic polyoxyethylene head. This architecture drives orientation at hydrophobic interfaces, where the hydrophobic chain interacts with air or container surfaces and the hydrophilic chain remains solvated in water. At concentrations above the critical micelle concentration (CMC), polysorbates self-assemble into micelles [153,173].

The stabilization of the drug product depends on surfactant concentration, temperature, ionic strength, and the physicochemical characteristics of the therapeutic protein. Importantly, polysorbate concentrations in commercial formulations are typically below 0.1 % (w/v), corresponding to 0.1-1 mg/mL. At these low levels, even minor degradation can significantly alter the equilibrium between monomeric and micellar species, leading to loss of functionality [61,107,171].

1.2.1.2 Degradation Pathways

Despite their stabilizing role, polysorbates are chemically and enzymatically prone to degradation. Degradation can proceed via two different pathways: (i) chemical degradation through hydrolysis or oxidation, and (ii) enzymatic hydrolysis catalyzed by residual host cell hydrolases. Both mechanisms have been documented in commercial biopharmaceutical products and can lead to serious quality and stability issues [48].

Under formulation-relevant conditions (typically pH 4.5-7.0), chemical degradation occurs through auto-oxidation. Oxidation is often initiated by reactive oxygen species or metal-ion contaminants (e.g. Fe^{2+} and Cu^{2+}), and leads to the formation of peroxides, aldehydes, and short-chain acids [212]. Factors accelerating chemical degradation include elevated temperature, light exposure, pH extremes, and trace metal catalysis. Formulation components such as buffers and amino acids may also influence degradation kinetics by modulating the microenvironment [179].

Enzymatic degradation has emerged as the dominant root cause of polysorbate instability in therapeutic mAb formulations. It is catalyzed by trace levels of host cell proteins (HCPs) that remain after downstream purification [32,106,131]. Because they originate from the CHO production host, their abundance and persistence depend strongly on purification efficiency [19].

Enzymatic hydrolysis generates free fatty acids and partial esters, leading to the gradual loss of surfactant molecules capable of forming micelles. Accumulated fatty acids, especially long-chain species such as palmitic and stearic acids, have low aqueous solubility and can crystallize into visible particles. These particles are often associated with secondary protein aggregation, reduced potency, or immunogenic responses [45,118,231].

1.3 Host Cell Proteins (HCPs)

During recombinant protein production, CHO cells secrete or release thousands of endogenous proteins collectively known as host cell proteins (HCPs). These proteins fulfill diverse cellular roles, including those related to growth, metabolism, protein synthesis, and folding [9,41]. HCPs can enter the process stream through normal secretion, stress-induced responses, or cell lysis occurring during upstream cultivation and harvest [19,91]. Although downstream purification processes are designed to remove the majority of these impurities, trace amounts can persist in the final drug substance.

The physicochemical diversity of the CHO proteome complicates impurity removal. HCPs vary widely in molecular weight (10-200 kDa), isoelectric point ($pI \approx 3-11$), hydrophobicity, and glycosylation [29,82,201,204]. This diversity can result in a broad elution behavior during ion-exchange, hydrophobic interaction, or mixed-mode chromatography. Furthermore, certain proteins display structural or surface-charge similarities to monoclonal antibodies, enabling co-elution or even reversible binding to the product [115].

Even at low concentrations, residual HCPs can negatively impact product quality by compromising stability, reducing potency, or triggering immunogenic responses [82,91]. Particularly problematic are enzymatically active HCPs, such as proteases and esterases, which may degrade the therapeutic protein or formulation excipients during storage. Regulatory authorities have set limits for residual HCPs in the final drug product, typically requiring concentrations below 100 parts per million (ppm) [82]. However, total HCP content alone is an insufficient indicator of risk, since individual species differ drastically in their biochemical activity. A single hydrolase or protease can compromise formulation stability even when total HCP concentrations meet specification limits [43]. This realization has shifted attention from bulk quantification to species-specific characterization of critical HCPs.

1.3.1 Mechanisms of Persistence in Downstream Processing

Downstream purification processes are designed to exploit general physicochemical differences between monoclonal antibodies and process-related impurities, such as charge, hydrophobicity, or molecular size. Despite the effectiveness of these strategies, certain host cell proteins consistently escape clearance and remain in the final drug substance. Their persistence is primarily attributed to two mechanisms: co-elution and product association [148].

Co-elution occurs when HCPs and therapeutic antibodies exhibit similar retention behavior on chromatographic resins, particularly in polishing steps such as ion exchange and mixed-mode chromatography [115]. In these cases, subtle differences in molecular properties are insufficient to achieve complete separation.

In addition to co-elution, some HCPs can associate with the product [114]. These interactions can occur through direct binding or complex formation, for example with high molecular weight species. The most frequently described form of product association is hitchhiking, in which an HCP reversibly binds to the surface of the antibody and effectively “rides along” during purification [2,114,160]. Unlike strong antigen-antibody complexes, hitchhiking interactions are weak and highly dynamic. Molecularly, hitchhiking is driven by electrostatic and hydrophobic complementarity. Hydrophobic loops or surface patches on HCPs can associate with hydrophobic patches on the antibody, while oppositely charged residues facilitate transient electrostatic attraction. In addition to direct antibody binding, HCPs may persist through association with protein aggregates or multiprotein networks formed during upstream processing or downstream purification. When antibodies self-associate or partially unfold, they expose hydrophobic or charged regions that can serve as nucleation sites for other proteins. HCPs trapped within such aggregates are physically shielded from chromatographic surfaces and can therefore co-purify with the product.

1.3.2 Polysorbate-Degrading Host Cell Proteins

A distinct group of host cell proteins with particular relevance in antibody manufacturing are polysorbate-degrading hydrolases. In recent years, enzymatic degradation of polysorbates has gained attention due to the formation of particles in drug products [45]. These particles were primarily identified as free fatty acids (FFAs), indicating hydrolytic cleavage of polysorbate ester bonds [48,102]. After excluding chemical hydrolysis, the degradation was traced back to hydrolases originating from CHO cells [32,71]. Despite being present at sub-ppm levels after downstream purification, these enzymes can remain catalytically active over time. This process has two major implications: first, the breakdown of polysorbates compromises their interfacial stabilizing function, increasing the risk of protein aggregation, especially under stress conditions; second, the accumulation of FFAs can exceed their solubility limits, resulting in the formation of subvisible and visible particles [172,231,232].

Consequently, thorough characterization and control of such degradation pathways are required to ensure product safety [118]. This includes the identification and

characterization of specific HCPs responsible for polysorbate degradation. Figure 1.4 illustrates the hierarchical classification of enzymes according to the Enzyme Commission (EC) system [138]. Among the seven major classes, hydrolases (EC 3) are highlighted because they catalyze hydrolytic cleavage of chemical bonds. Within this group, the subclass 3.1 acting on ester bonds is particularly relevant, as it includes enzymes capable of cleaving ester linkages such as those found in polysorbates.

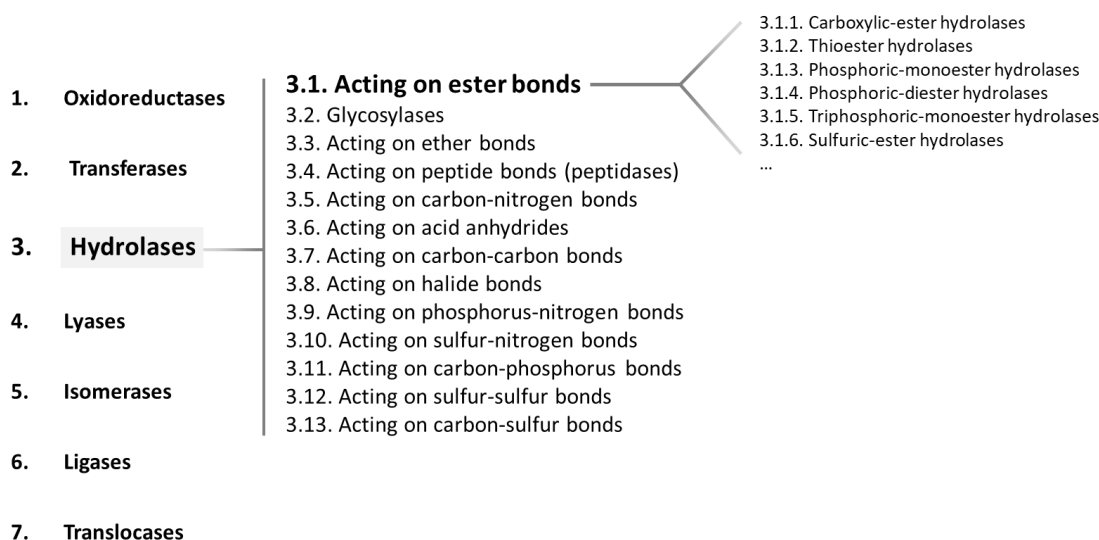


Figure 1.4: Classification of enzymes according to the Enzyme Commission (EC) system with a focus on hydrolases acting on ester bonds.

Historically, the phospholipase B-like 2 (PLBL2) protein was considered the main candidate for degrading polysorbates. PLBL2 was frequently detected in purified antibody formulations and shares sequence homology with lipases, suggesting potential esterase activity. Moreover, early studies using recombinant PLBL2 reported measurable hydrolytic activity against polysorbates, apparently confirming its enzymatic role [43]. However, this interpretation was later proven incorrect. Detailed biochemical investigations revealed that the previously observed activity did not originate from PLBL2 itself but from a trace contaminant present in the recombinant protein preparation. When highly purified PLBL2 was tested, no polysorbate-degrading activity was detected. This finding was further supported by knockout studies in CHO cells, which demonstrated that the removal of the PLBL2 gene had no measurable effect on polysorbate stability in antibody formulations [237]. These results conclusively show that PLBL2, despite its lipase-like sequence and frequent detection, does not catalyze polysorbate hydrolysis.

The PLBL2 case exemplifies a broader challenge in the interpretation of host cell protein data: the presence of a protein does not imply functional relevance. Proteomic identification alone cannot differentiate between enzymes that are truly active and those that are either intrinsically inactive or rendered non-functional during processing. In some cases, proteins may lose catalytic activity through misfolding or aggregation, effectively inactivating their active sites. This distinction between detection and functionality is essential

for accurate risk assessment and underscores the need for functional validation of potential polysorbate-degrading enzymes.

1.4 Analytical Approaches for Polysorbate Stability and HCP Characterization

Figure 1.5 summarizes the analytical approaches applied throughout this dissertation to investigate the behavior of polysorbate-degrading HCPs and their activity against polysorbate. Starting from different formulated monoclonal antibody products, candidate HCPs were identified and subsequently expressed and purified to enable controlled mechanistic studies. Their characterization was performed along two complementary dimensions.

The first dimension focuses on enzymatic activity, combining fluorescence-based assays, including the fluorescence micelle assay (FMA) and the 4-methylumbelliferone assay (MU4), with direct analysis of polysorbate degradation profiles using reverse-phase ultra-performance liquid chromatography coupled to mass spectrometry (RP-UPLC-MS) to obtain enzyme-specific fingerprint patterns. These fingerprints also provided the basis for data-driven classification approaches.

The second dimension addresses downstream-relevant behavior, including high-throughput binding screenings to study partitioning behavior (K_p screenings) and biophysical interaction analysis using biolayer interferometry to quantify transient interactions between HCPs and monoclonal antibodies. Together, these analytical approaches provide complementary perspectives on polysorbate-degrading host cell hydrolases, from identifying candidate enzymes to resolving their specific degradation patterns to gaining mechanistic insights into their chromatographic behavior and their transient interactions with monoclonal antibodies. The following sections introduce these analytical approaches in more detail.

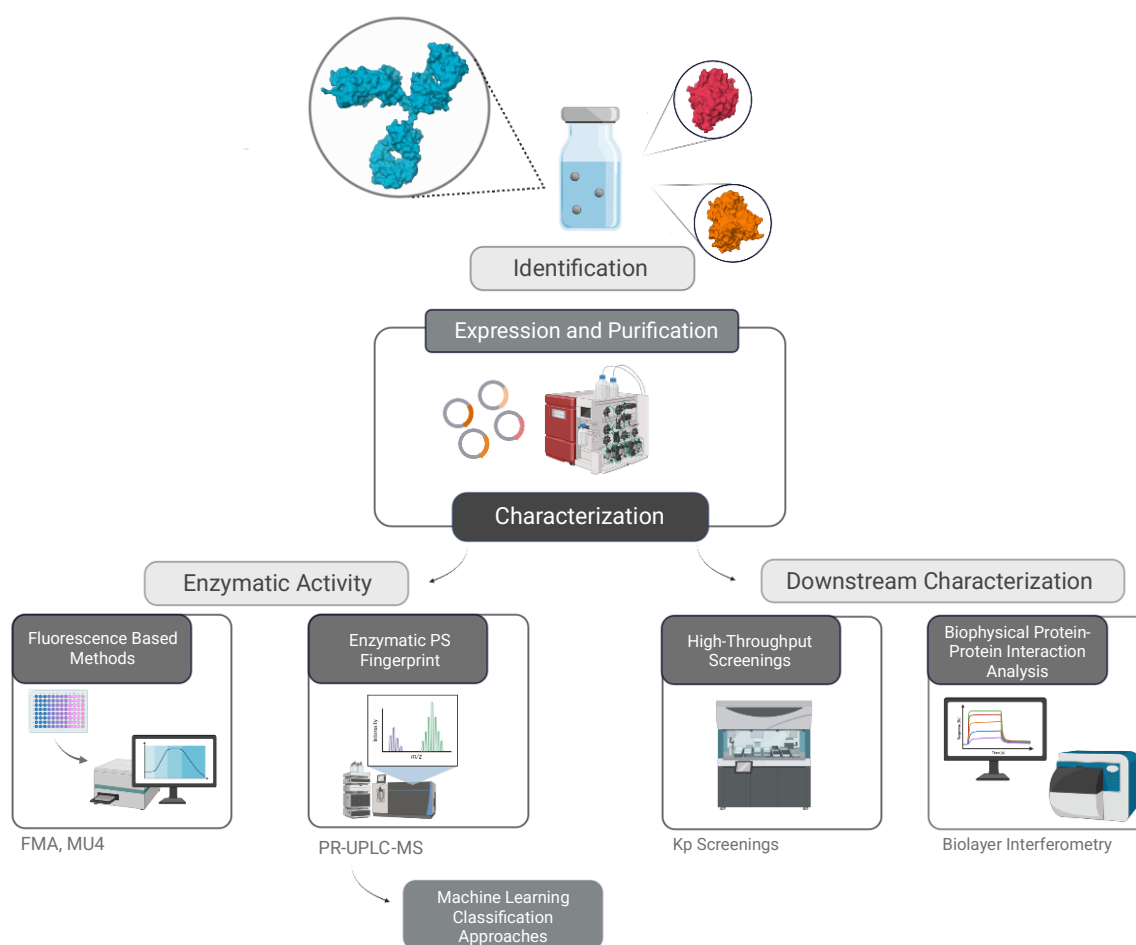


Figure 1.5: Schematic summary of the workflow applied to investigate polysorbate degradation and host cell protein behavior. Candidate enzymes were first identified in formulated monoclonal antibody (mAb) products and subsequently recombinantly expressed and purified. Their enzymatic activity was assessed using fluorescence-based assays, including the fluorescence micelle assay (FMA), the 4-methylumbelliferone assay (MU4), and by direct analysis of polysorbate degradation profiles using reverse-phase ultra-performance liquid chromatography coupled to mass spectrometry (RP-UPLC-MS). In parallel, high-throughput partitioning screenings (Kp screenings) and biolayer interferometry (BLI) were employed to investigate chromatographic behavior and transient mAb-HCP interactions relevant for downstream processing. Together, these methods provide complementary perspectives on the identity, activity, and process behavior of polysorbate-degrading hydrolases. (Created using BioRender.com)

1.4.1 Mass Spectrometry-Based Identification of PS Degrading HCPs

Different analytical strategies have been developed to identify host cell proteins (HCPs) that catalyze polysorbate (PS) degradation. Since hydrolytic HCPs typically occur at trace levels (often below 1 ppm relative to the therapeutic antibody), their identification remains highly challenging. Therefore, most analytical workflows start with steps that reduce the dynamic range and enrich the low-abundance fraction prior to mass spectrometry (MS) analysis [54].

A common first step is the removal of the antibody, either by Protein A chromatography or by filtration using molecular weight cut-off membranes, such as 50 kDa filters. The idea is to separate smaller proteins from the large antibody molecule (~150 kDa) by retaining the antibody in the retentate and collecting smaller proteins in the filtrate [29]. However, this approach is not well suited for identifying PS-degrading enzymes, as many relevant proteins have molecular weights close to or above the cut-off threshold.

More effective enrichment strategies include ProteoMiner and anti-HCP affinity chromatography. ProteoMiner uses a combinatorial peptide ligand library to reduce the dynamic range of protein concentrations by saturating high-abundance proteins and retaining low-abundance species [18]. When combined with native or limited digestion, ProteoMiner has been shown to significantly increase the number of detectable HCPs and improve sensitivity down to the ppb level [30,236]. Anti-HCP affinity capture selectively isolates HCPs using polyclonal antibodies [206]. Chromatographic methods such as size-exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC) further support enrichment by separating proteins based on size or hydrophobicity [224,241].

Sample preparation typically involves limited tryptic digestion under non-denaturing conditions, favoring proteolysis of more accessible HCPs, whereas the compact, disulfide-stabilized IgG structure exhibits reduced susceptibility to enzymatic cleavage [84]. Additives such as sodium deoxycholate improve solubilization and reduce protein-protein interactions. Peptide-level fractionation can further reduce sample complexity and enhance detection of low-abundance peptides [54].

Mass spectrometric analysis can be performed using different acquisition approaches. In data-dependent acquisition (DDA), the most intense precursor ions are selected for fragmentation. This allows broad proteome coverage but may miss low-abundance peptides due to stochastic sampling. In data-independent acquisition (DIA), all ions within defined m/z windows are fragmented. This results in more consistent and comprehensive peptide detection. DIA is therefore well suited for detecting low-abundance enzymes, especially when combined with library-free analysis [54,194]. For quantitative analysis, targeted MS methods such as parallel reaction monitoring (PRM) and selected reaction monitoring (SRM), commonly referred to as multiple reaction monitoring (MRM), are used. These approaches provide high sensitivity and specificity, especially when stable isotope-labeled internal standards are included [54,118].

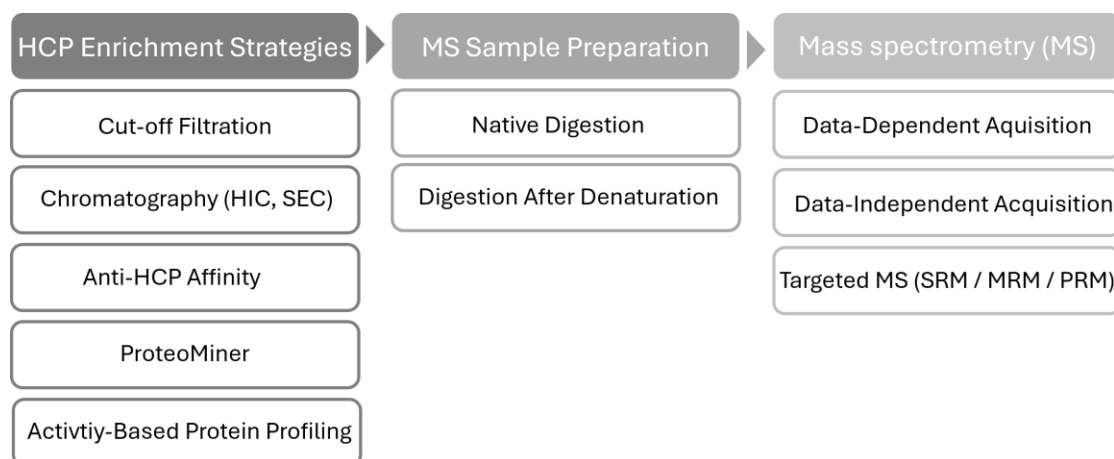


Figure 1.6: Overview of mass spectrometry (MS)-based strategies for the identification and quantification of polysorbate-degrading host cell proteins (HCPs). The workflow consists of three main components: HCP enrichment to concentrate low-abundance proteins; sample preparation to reduce matrix complexity and improve detection sensitivity and mass spectrometric analysis. MS analysis includes discovery-oriented approaches such as data-dependent acquisition (DDA) and data-independent acquisition (DIA) for broad identification, as well as targeted MS methods (SRM, MRM, PRM) for quantification of selected HCP species. Modified from [54].

Mass spectrometry-based identification yields valuable information on the presence and relative abundance of potential PS-degrading enzymes. However, it does not confirm catalytic activity. Functional assays are needed to determine whether the detected enzymes are active under formulation-relevant conditions. A widely used technique for this purpose is Activity-Based Protein Profiling (ABPP) [121]. ABPP employs small molecule probes that covalently bind to nucleophilic residues within the active site of enzymes (most commonly the catalytic serine or cysteine, depending on the enzyme class). These probes carry reporter or affinity tags (e.g. biotin) for subsequent enrichment and MS analysis. MS analysis then allows the distinction between active and inactive enzymes. This is highly effective for detecting active hydrolases in complex mixtures. However, ABPP does not reveal substrate specificity, as the probes target conserved catalytic motifs. Consequently, all active serine or cysteine hydrolases are labeled, including enzymes unrelated to polysorbate degradation, such as proteases [39,117].

Therefore, complementary approaches such as recombinant expression and direct functional testing of individual candidate HCPs remain essential to confirm polysorbate-degrading activity. These methods allow for a more detailed characterization, including substrate specificity, reaction kinetics, and pH dependence under formulation-relevant conditions. Together, the combination of proteomic identification and functional validation provides a basis for a risk assessment of polysorbate-degrading HCPs in therapeutic protein formulations.

1.4.2 Downstream Characterization of Host Cell Proteins

Downstream characterization of HCPs relies traditionally on generic HCP ELISAs, which quantify the total impurity content in process intermediates. This is essential for monitoring purification performance. However, these assays detect only immunoreactive proteins and provide no information on the identity or concentration of individual species [118]. Consequently, ELISAs are poorly suited for assessing the behavior of specific HCPs, such as enzymatically active polysorbate hydrolases. For this group of proteins, analytical characterization during downstream development is very challenging. To date, no commercial ELISA exists for individual polysorbate-degrading hydrolases, largely due to the lack of suitable antibodies and the high similarity of many lipase-like proteins [54]. Mass spectrometry-based approaches can identify individual HCPs [19,144,149]. However, these methods are low-throughput, and the challenge is further amplified in samples where high antibody concentrations suppress signals from low-abundance HCPs [54]. As a result, MS-based workflows are challenging for the large number of samples typically generated during downstream development. Nevertheless, downstream process development increasingly depends on analytical strategies that characterize how specific critical HCPs behave during purification rather than solely detect their presence.

High throughput binding screening approaches can support this need by characterizing the HCP binding behavior during purification. From these experiments, partition coefficients (K_p) can be determined for both the product and individual impurities [139]. This describes quantitatively how strongly each species interacts with the stationary phase relative to the mobile phase and thus provides a direct measure of binding propensity under controlled variations of pH and conductivity. By compiling K_p data across multiple experimental conditions, these screenings can provide an indication of which HCPs exhibit physicochemical features that promote co-elution with antibodies. Furthermore, this approach may support the identification of an operating window that maximizes hydrolase removal.

When combined with orthogonal analytical data derived from chromatography column experiments, high throughput binding screenings enhance understanding of HCP partitioning and support the development of purification conditions aimed at reducing impurity persistence.

1.4.3 Biophysical Interaction Analysis (Biolayer Interferometry)

In addition to their chromatographic behavior, a subset of HCPs persists during downstream processing because they interact with the therapeutic antibody [200]. These associations can enable HCPs to remain bound to the product through affinity and polishing steps, even when their intrinsic physicochemical properties would otherwise predict efficient clearance. This phenomenon, commonly referred to as hitchhiking, has gained increasing attention as a mechanistic explanation for why certain impurities repeatedly appear in highly purified monoclonal antibody preparations [114,200].

Such protein-protein interactions are governed by the dynamic equilibrium between association and dissociation events. The strength and kinetics of these interactions are

described by rate constants for association and dissociation, as well as by the equilibrium dissociation constant, which reflects the overall affinity. Affinity values can span several orders of magnitude: High-affinity antigen-antibody interactions typically fall in the low-nanomolar range, whereas nonspecific or transient interactions often lie in the micromolar range [154].

A variety of biophysical methods exist to measure protein-protein interactions [143,180,181,228]. Surface plasmon resonance (SPR) monitors refractive index changes at a sensor surface and offers high sensitivity and kinetic analysis. Biolayer interferometry (BLI) similarly detects mass accumulation on a sensor surface but operates in a dip-and-read format without microfluidics. Microscale thermophoresis (MST) measures molecular movement in temperature gradients and enables affinity determination in free solution, although it typically lacks kinetic resolution. Isothermal titration calorimetry (ITC) provides thermodynamic parameters but requires relatively high sample quantities and is less suited for weak interactions. Additional techniques such as dynamic light scattering (DLS) or analytical size-exclusion chromatography (SEC) can detect larger complexes or aggregation but cannot quantify transient binding events.

Among these methods, biolayer interferometry is particularly well suited for the analysis of HCP-antibody interactions relevant to downstream processes. As multiple sensors can be run in parallel, BLI additionally supports high-throughput kinetic screening, which is particularly advantageous when investigating multiple HCP candidates, various antibody formats, or a range of process relevant buffer conditions. In BLI, white light is reflected from an internal reference layer and an outer layer functionalized with an immobilized ligand (e.g., an antibody). When an analyte protein binds to the surface, the optical thickness of the biolayer changes, resulting in a detectable wavelength shift ($\Delta\lambda$) (Figure 1.7). This shift is monitored in real time and can be visualized in sensorgrams that capture both association and dissociation phases [1,7,181,193].

The equilibrium dissociation constant, K_D , expresses the affinity of the interaction and is defined as:

$$K_D = \frac{k_{off}}{k_{on}}$$

Where k_{on} is the association rate constant and k_{off} the dissociation rate constant. Lower K_D values indicate higher affinity. The interaction is commonly modeled using a 1:1 Langmuir binding model, assuming independent and equivalent binding sites. Several kinetic variants of the Langmuir binding model exist. To obtain robust kinetic parameters, measurements at several concentrations are typically evaluated using a global fit, in which a single set of kinetic constants is optimized to describe all association and dissociation traces simultaneously. Full fitting of the 1:1 model typically optimizes three parameters: k_{on} , k_{off} , and R_{max} , the maximum binding capacity, enabling determination of both binding affinity (K_D) and kinetic parameters.

Because BLI measures mass accumulation directly at the sensor surface and enables real-time monitoring under flexible assay conditions, it is well suited for detecting weak and

transient binding events relevant to downstream processing. In this study, BLI was selected to investigate the interactions between polysorbate-degrading hydrolases and monoclonal antibodies.

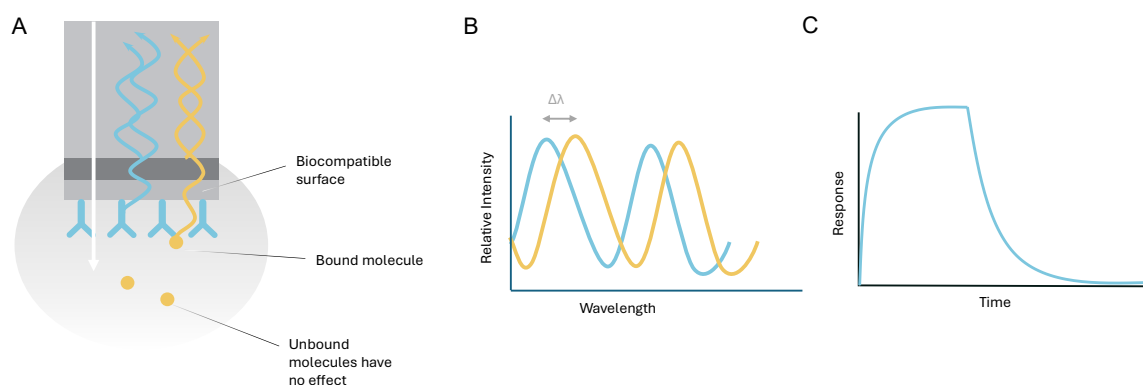


Figure 1.7: Principle of biolayer interferometry for real-time binding analysis. A: Incident light passes through an optical layer and is reflected from two surfaces: a fixed reference layer and a biocompatible layer where molecules of interest are immobilized. B: Binding of analytes changes the optical thickness at the biocompatible surface, which causes a measurable wavelength shift ($\Delta\lambda$) in the interference pattern. This wavelength shift is recorded over time as a sensorgram, enabling determination of binding kinetics.

1.4.4 Analytical Techniques to Investigate Polysorbate Degradation

A broad range of analytical techniques exist to investigate polysorbate degradation [25,53,54,60,118,158]. The analytical landscape is summarized in Figure 1.8, which illustrates both direct and indirect approaches. Indirect assays, typically fluorescence-based, report overall changes in polysorbate concentration or general esterase activity, whereas direct analysis can resolve individual polysorbate species and thereby offer deeper information about enzymatic degradation pathways. The following section outlines the principle, strengths, and limitations of the most commonly used methods to investigate PS degradation.

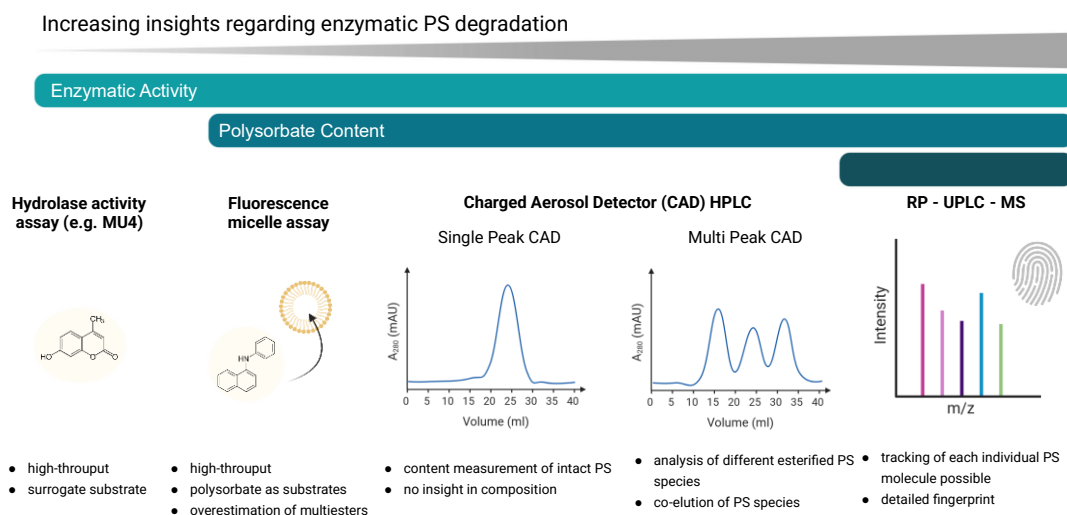


Figure 1.8: Overview of analytical techniques to investigate polysorbate degradation. Hydrolase activity assays (e.g., MU4) are indirect fluorescence-based methods using synthetic ester substrates as a surrogate for PS to assess general hydrolytic activity. MU4 refers to 4-methylumbelliferone, which is released upon ester bond cleavage and can be detected via fluorescence. The fluorescence micelle assay (FMA) monitors PS concentration via micelle formation and dye incorporation. The fluorescent dye NPN (N-phenyl-1-naphthylamine) partitions into micelles, which increases the fluorescent signal. Degradation of polysorbate reduces micelle concentration and fluorescence. Charged Aerosol Detection (CAD) coupled to High-Performance Liquid Chromatography (HPLC) enables quantification of total PS content. In single-peak CAD, all PS species co-elute, whereas multi-peak CAD uses optimized gradients to partially resolve esterified species (mono-, di-, multiesters). Reverse-phase ultra-performance liquid chromatography combined with mass spectrometry (RP-UPLC-MS) provides the most detailed analysis by identifying individual PS subspecies based on their mass-to-charge ratio (m/z), generating a so-called “polysorbate fingerprint”. (Created using BioRender.com).

MU4 Hydrolytic Activity Assay

The 4-methylumbelliferone (MU4) hydrolytic activity assay is an indirect method to assess the hydrolytic activity of enzymes against ester bonds, serving as a surrogate readout for potential polysorbate-degrading activity [12,87]. The assay is based on synthetic fluorogenic substrates consisting of 4-methylumbelliferone (MU4) esterified to fatty acids of varying chain length (e.g., C8, C10, C12, C16). Upon enzymatic cleavage of the ester bond, free MU4 is released, which can be detected via fluorescence. Therefore, fluorescence over time allows assessment of hydrolytic activity.

While the MU4 assay offers simple handling and high throughput, it has limitations when used to assess polysorbate degradation. The surrogate substrates do not reflect the structural heterogeneity of polysorbates, particularly with respect to different head groups and different esterified species. As a result, activity levels in MU4 assays may not correspond to activity towards polysorbates in a formulation. The assay allows a first indication of

general hydrolase activity but does not allow reliable conclusions about the extent of polysorbate hydrolysis.

Fluorescence micelle assay (FMA)

The fluorescence micelle assay (FMA) is a widely used method for monitoring the concentration of polysorbates in protein formulations [60,120]. This method relies on the ability of non-ionic surfactants to form micelles above their critical micelle concentration (CMC). The fluorescent dye N-phenyl-1-naphthylamine (NPN) partitions into the hydrophobic core of micelles, increasing its fluorescent signal. As polysorbates degrade, micelle concentration decreases. This results in a proportional reduction in fluorescence intensity.

The FMA is fast and applicable to high-throughput formats. However, like other indirect assays, it has certain limitations. Different polysorbate species have different critical micelle concentrations (CMC). Monoesters form micelles less efficiently than diesters or multiesters and therefore contribute less to the assay readout. This biases the assay toward overrepresenting the loss of multiester fractions and may underestimate hydrolysis events that primarily target monoester chains [45,53,120].

CAD-based HPLC (single-peak and multi-peak CAD)

High-performance liquid chromatography (HPLC) methods are widely used for polysorbate analysis in the pharmaceutical industry. Because polysorbates lack a chromophore, UV detection is not feasible. Therefore, other detectors such as evaporative light scattering (ELSD), charged aerosol detection (CAD), and mass spectrometry (MS) are employed [136,158,183].

CAD is a mass-sensitive detector that enables the detection of analytes regardless of their UV activity [44,183]. The eluent from the HPLC is first converted into a fine mist of droplets. These droplets are dried and subsequently charged, and the detector measures the amount of charge, which is directly proportional to the mass of the analyte. This makes CAD particularly suitable for substances like polysorbates that do not absorb UV light.

In single-peak CAD, all polysorbate species co-elute and form one broad peak that represents the total polysorbate content. This is typically achieved using step elution or non-optimized gradient conditions that do not resolve subspecies. In multi-peak CAD, optimized gradient elution and column chemistry partially resolve subspecies based on hydrophobicity differences. This results in multiple peaks representing mono-, di-, and multiester species. However, resolution remains limited because many subspecies have similar polarity and tend to co-elute.

Most methods use reverse-phase columns such as C18 [183]. The mobile phase typically consists of water and acetonitrile. Gradients are applied to gradually increase organic content, which reduces retention of hydrophobic species and enables partial separation.

Single-peak CAD is simple and robust for quantifying total polysorbate content but provides no insight into subspecies or degradation pathways. Multi-peak CAD provides more structural information by partially resolving esterification states, but the separation

remains inherently limited because many subspecies share very similar polarity and therefore tend to co-elute.

Polysorbate Fingerprinting via RP-UPLC-MS

Reverse-phase ultra-performance liquid chromatography coupled to mass spectrometry (RP-UPLC-MS) has become the gold standard for detailed analysis of polysorbate degradation. RP-UPLC separates polysorbate molecules primarily based on hydrophobicity, using C8 or C18 stationary phases and optimized gradients of water, acetonitrile, and methanol [79]. Coupling RP-UPLC with a mass spectrometer, such as a single-quadrupole detector (QDa), enables precise identification of individual subspecies by their mass-to-charge (m/z) ratios [168]. The resulting dataset forms a “polysorbate fingerprint,” which is a detailed profile of all detectable PS subspecies. Monitoring changes in this fingerprint over time provides insight into degradation pathways. Compared to conventional methods such as RP-HPLC with CAD or ELSD, RP-UPLC-MS offers superior specificity and sensitivity, resolving co-eluting species and enabling quantitative assessment of individual subspecies.

As polysorbate degradation patterns comprise multiple polysorbate species and change over time, their interpretation becomes increasingly complex. In such cases, data-driven classification methods can support the assignment of degradation profiles to specific hydrolases. This concept is applied in Chapter 7 as an additional analytical tool to aid functional enzyme identification.

1.5 Mitigation Strategies

1.5.1 Cell Line Engineering

Cell line engineering has emerged as a powerful approach for optimizing biopharmaceutical production cell lines [56,101,109]. In this context, one particularly effective strategy to mitigate polysorbate degradation is to tackle the issue at its origin: the production cell line. By reducing the number of active enzymes secreted into the culture supernatant, the risk of polysorbate hydrolysis can be significantly reduced [32]. Cell line engineering strategies generally fall into two categories: gene knockouts, which aim to eliminate the functional protein, and gene knockdowns, which reduce protein expression to a lower level [56]. Both approaches can be applied to individual hydrolases or simultaneously to multiple enzymes [32,216].

Gene knockout approaches aim to permanently disrupt the coding sequence of a target gene, resulting in the complete loss of the functional protein. This is commonly achieved using genome-editing tools such as zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), or RNA-guided nucleases such as the CRISPR/Cas9 system, based on clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated protein 9 [34,59,142,175].

The Cas9 is an RNA-guided endonuclease and the primary component of the CRISPR/Cas9 genome editing system. It mediates site-specific DNA cleavage [133]. Targeted recognition is achieved through a single-guide RNA (sgRNA), which combines a trans-activating CRISPR RNA (tracrRNA) and a CRISPR RNA (crRNA). The crRNA provides sequence complementarity to the genomic target, ensuring specificity. For cleavage to occur, the target site must be adjacent to a protospacer adjacent motif (PAM), typically represented as NGG, where N denotes any nucleotide and G denotes guanine. After binding, the Cas9-sgRNA complex creates a double-strand break three base pairs upstream of the PAM. The resulting break is primarily repaired by non-homologous end joining. This error-prone repair process often results in insertions or deletions (indels), causing frameshifts and gene disruption [34,88,150]. Consequently, the CRISPR/Cas9 system is particularly useful for engineering cell lines with targeted gene knockouts such as PS hydrolases.

A widely discussed example in the context is lipoprotein lipase (LPL). LPL is a secreted lipase with known activity against ester substrates. Studies have shown that cell lines lacking LPL exhibit improved polysorbate stability [32]. LPL knockout thus serves as proof-of-concept that targeted removal of single hydrolases can have a substantial impact on polysorbate degradation risk.

Despite their potential, gene knockouts must be implemented carefully, as some hydrolases might have physiological roles that are essential for the cell. Complete loss of such genes might therefore adversely affect cell growth or productivity. Moreover, CHO cells often express multiple hydrolases with overlapping substrate specificities, so targeting one enzyme might lead to upregulation of another enzyme.

Removing several enzymes at once increases the risk of unexpected phenotypes. For these reasons, knockout strategies require detailed prior characterization [189]. This includes the assessment of growth kinetics, productivity, and critical quality attributes.

An additional practical consideration in implementing specific hydrolase knockouts lies in the risk of violating existing intellectual property rights, as certain gene targets or knockout combinations may be covered by patents [54]. Therefore, a thorough freedom-to-operate assessment is essential before such engineered cell lines can be deployed in commercial production.

In contrast to knockout, gene knockdown strategies aim to reduce rather than abolish the expression of a target gene. This is typically achieved using RNA interference-based tools such as small interfering RNAs, short hairpin RNAs, or artificial microRNAs. These molecules guide the RNA-induced silencing complex (RISC) to target mRNA copies, promoting their degradation or preventing translation [73,85,162].

Knockdown strategies are particularly attractive when complete loss of a hydrolase is not desired, for example, if the enzyme plays a role in essential metabolic pathways, or if partial residual activity is sufficient to maintain cellular functions. Moreover, RNA-based approaches can be multiplexed more easily than DNA knockouts [216]. However, knockdown

effects are typically incomplete and variable, depending on expression levels, vector integration sites, and potential epigenetic silencing over time [15]. As a result, knockdown-based mitigation may reduce but not abolish polysorbate degradation.

1.5.2 Bioprocess Optimization

Whereas cell line engineering aims to reduce hydrolase expression at the genetic level, bioprocess optimization focuses on minimizing the release and persistence of these enzymes during cell culture and purification.

Upstream cultivation conditions can have a significant influence on the quantity and composition of host cell proteins entering the downstream process [72]. CHO cells secrete different endogenous proteins, and the type and number of HCPs, including polysorbate-degrading hydrolases, can vary widely depending on culture parameters, cell viability, or even the cell culture medium [105,220].

One of the strongest determinants of HCP burden is cell viability at harvest [86]. As viability declines, cells release significant amounts of intracellular enzymes. Therefore, high cell viability at the end of the production process is generally desirable. However, with regard to polysorbate-degrading hydrolases, high cell viability only affects enzymes that predominantly reside intracellularly.

Furthermore, CHO clones differ substantially in their basal expression of hydrolases, even when derived from the same transfection pool [72,203]. During clone selection, primary screenings usually focus on productivity and product quality. However, the investigation of transcriptomic heterogeneity between different clonal production cell line candidates might enable the selection of superior cell lines with favorable HCP profiles. This, however, depends on the availability of highly precise and sensitive MS-based quantification techniques that allow rapid detection of specific HCP species in the harvested cell culture supernatant.

After the cell culture harvest, the overall HCP load can be lowered by applying an acidic precipitation step, for instance using caprylic acid [22,69]. This promotes the removal of a fraction of host cell proteins from the harvested cell culture supernatant. Such an approach, however, is only feasible if the therapeutic protein remains stable and does not co-precipitate under these acidic conditions.

Downstream purification plays a central role in mitigating the carryover of polysorbate-degrading host cell proteins. Effective mitigation strategies in downstream processing are therefore essential to maintain drug product quality and shelf life.

A typical process for monoclonal antibody purification consists of three major chromatographic steps: Protein A capture, flow-through polishing (e.g. anion exchange or mixed-mode chromatography) and bind/elute polishing (e.g. cation exchange chromatography) [27,122]. Each step contributes to impurity clearance, but certain HCPs persist due to mechanisms such as hitchhiking, where proteins form interactions with antibodies or co-

purify as part of aggregate complexes [54,114]. These interactions make complete removal challenging and necessitate targeted process adaptations.

Several strategies have been developed to enhance HCP clearance during downstream processing. Optimized wash buffers during Protein A chromatography are often developed to enhance clearance early in the process [35,185].

Following capture, the product undergoes low-pH viral inactivation, usually around pH 3.5, followed by neutralization. This pH shift induces precipitation, which significantly reduces impurity levels. The extent of precipitation depends on both the neutralization pH and the buffer system. For example, glycine-based buffers promote stronger precipitation than acetate systems due to lower conductivity [112].

After depth filtration, during which the precipitates are removed, usually two polishing steps are performed. Flow-through chromatography using anion exchange (AEX) or mixed-mode resins is commonly employed to bind negatively charged and hydrophobic impurities while the antibody remains in the flow-through. Loading conditions such as high pH and low conductivity are optimized to maximize HCP removal [97,98]. Subsequent bind/elute steps, typically using cation exchange (CEX) chromatography, provide additional clearance and can be fine-tuned through gradient elution to separate impurities and aggregates [115,191]. In some cases, hydrophobic interaction chromatography (HIC) is introduced as an alternative polishing step [108].

The overall downstream purification sequence strongly influences HCP clearance and, consequently, polysorbate stability in the final drug product. Early removal of aggregates and HCP-rich complexes, combined with optimized polishing strategies, significantly reduces the risk of enzymatic degradation. While downstream processing can achieve HCP levels near the detection limit of general HCP ELISAs, residual hydrolases may still persist [71].

1.5.3 Formulation Strategies

Formulation strategies to mitigate enzymatic polysorbate degradation focus on controlling environmental factors and exploring excipient alternatives [17,171,231]. One influential parameter is formulation pH, which affects both chemical and enzymatic degradation pathways. Hydrolytic enzymes exhibit distinct pH optima, and even small shifts can alter hydrolysis rates [12,60]. While most commercial monoclonal antibody formulations employ a pH between 5.0 and 6.0 to balance protein stability and minimize chemical degradation, this range does not necessarily suppress enzymatic activity [60,192]. Studies indicate that hydrolase-mediated PS hydrolysis can occur across a broad pH spectrum, suggesting that pH adjustment alone is insufficient as a standalone mitigation measure.

Temperature is another critical factor [54,107,235]. Elevated storage temperatures accelerate both oxidative and hydrolytic degradation, increasing free fatty acid (FFA) and particle formation. Consequently, storage conditions at 2-8 °C can substantially slow these processes. However, temperature control cannot fully prevent enzymatic degradation if residual host cell proteins remain active in the formulation.

Formulation composition also modulates the solubility and aggregation behavior of degradation products. Adjusting ionic strength, buffer species, and co-solvent composition can improve FFA solubility and reduce particle risk [54,67].

A major challenge arises in high-concentration mAb formulations (>100 mg/mL), increasingly required for subcutaneous administration [184,210]. Ultrafiltration/diafiltration steps proportionally concentrate any co-purified HCPs, thereby amplifying enzymatic degradation risk.

To address these limitations, alternative surfactants are under investigation. Poloxamer (often Poloxamer 188) is one of the very few non-ionic surfactants besides polysorbates (Polysorbate 20 and 80) that has been reported in commercial biologics [17]. Its use is less common than that of polysorbates, but it appears in certain formulations, mainly for monoclonal antibodies. However, P188 degradation can occur under oxidative stress, particularly in the presence of trace metals and elevated temperatures [31,209].

Other surfactants are to date not used in approved biologics because of regulatory familiarity, safety profiles, and compatibility with proteins [23]. Poloxamer and polysorbates dominate because they have well-established toxicology, regulatory acceptance, and a long history of use in biopharmaceutical manufacturing.

2 Contribution of this Thesis

2.1 Research Proposal

Biopharmaceuticals have become one of the most important classes of therapeutic products in recent years, offering treatments for a wider range of diseases. Ensuring their stability is a critical challenge as protein aggregation and interfacial stress can compromise product quality and safety. To mitigate these risks, polysorbates are widely used as essential excipients because of their stabilizing properties. However, the degradation of polysorbates in drug products has become a major challenge for the biopharmaceutical industry.

Over the past decade, it has become evident that polysorbate degradation is predominantly driven by host cell hydrolases originating from CHO production systems. Even at sub-ppm levels, they can cleave the ester bonds of polysorbates, leading to loss of excipient functionality and formation of free fatty acids, which can result in particle formation in the final drug product.

This issue is further amplified by rising upstream titers, which increase the absolute amount of host cell proteins entering downstream processing, and by higher antibody concentration in final formulations. Furthermore, the shift of the therapeutic landscape toward complex biologics such as bispecific antibodies, trispecific formats and Fc-fusion proteins challenges established purification processes and may facilitate the persistence of specific hydrolases.

Despite the industrial relevance of this problem, several fundamental knowledge gaps remain. Classical HCP analytics such as generic ELISAs provide only bulk impurity levels and fail to detect low-abundance hydrolases or distinguish between active and inactive forms. Proteomic identification alone does not reveal enzymatic activity, while functional polysorbate assays lack information about enzyme identity. Even when candidate hydrolases are identified, it often remains unclear whether they actively contribute to polysorbate degradation and why certain enzymes persist through purification while others are efficiently removed. These limitations hinder the development of targeted mitigation strategies.

The aim of this dissertation is therefore to establish a deeper understanding of polysorbate degrading hydrolases in CHO-based manufacturing by combining complementary analytical, biochemical, and process-relevant perspectives.

The first part of the project will focus on identifying polysorbate degrading hydrolases present in formulated monoclonal antibody products by combining proteomic analysis with

recombinant expression and purification of candidate enzymes. Their characterization will provide a defined set of enzymes that contribute to polysorbate degradation under formulation-relevant conditions. Building on this starting point, a second study will examine how these hydrolases behave during downstream purification polishing steps. High-throughput binding screenings will be used to evaluate their binding behavior in comparison to different model antibodies, with the aim of understanding why certain hydrolases persist. The third study will investigate transient interactions between hydrolases and monoclonal antibodies using biolayer interferometry. These measurements will not only characterize the affinities of the specific HCPs to different antibodies but also help clarify whether reversible HCP-mAb associations contribute to hydrolase carryover and co-elution during purification.

While these studies will clarify identity and process-related behavior, two further investigations will focus on the enzymatic activity against polysorbate in more detail.

Using RP-UPLC-MS, individual hydrolases and their polysorbate degradation products will be investigated to explore whether enzyme-specific degradation fingerprints can be observed. This study will assess whether distinct substrate preferences exist among different polysorbate hydrolases.

The final study will then expand this concept by exploring machine-learning-based classification of those fingerprints with the goal of linking distinct polysorbate cleavage patterns to individual hydrolases. This study aims to evaluate whether the pattern of polysorbate cleavage is sufficient to distinguish between individual hydrolases and thereby reveal which enzyme is functionally responsible for degradation in a given formulation.

By combining these perspectives, the dissertation aims to move beyond the traditional descriptive view of HCP impurities toward a mechanistic understanding of how individual hydrolases behave during the bioprocess. It connects the origin, persistence and activity of polysorbate degrading enzymes within an integrated framework that informs targeted mitigation strategies across the manufacturing chain. This knowledge will support the development of targeted mitigation strategies, including cell line engineering and tailored downstream purification, and ultimately contribute to the design of more robust biopharmaceutical production platforms.

2.2 Manuscript Overview

This thesis aims to provide a mechanistic understanding of polysorbate degrading host cell hydrolases by integrating analytical, biochemical, and downstream-focused perspectives across five research articles. Together, these studies establish a systematic framework from identifying relevant enzymes to characterizing their persistence during purification to resolving their functional activity. This chapter provides a brief overview of these publications and their contributions. Four manuscripts have been accepted for publication in well-established, peer-reviewed international journals, and one manuscript is currently under review.

Chapter 3: Illuminating a biologics development challenge: systematic characterization of CHO cell-derived hydrolases identified in monoclonal antibody formulations

Melanie Maier*, Linus Weiss*, Nikolas Zeh, Valerie Schmieder-Todtenhaupt, Alireza Dehghani, Marius Nicolaus Felix, Daniel Heinzelmann, Benjamin Lindner, Moritz Schmidt, Joey Studts, Patrick Schulz, Bernd Reisinger, Kerstin Otte, Matthias Franzreb, Daniel Lakatos, Simon Fischer

(*contributed equally)

mAbs (2024), doi: 10.1080/19420862.2024.2375798

The research article presented in Chapter 3 establishes the foundation for this work by identifying and characterizing a set of CHO-derived hydrolases capable of degrading polysorbates under formulation-relevant conditions. The work employs proteomic identification of CHO hydrolases in monoclonal antibody products, their recombinant expression, sub-cellular localization, expression profiling and activity screening to narrow the broad CHO proteome to a defined group of enzymes active against polysorbate. This interdisciplinary approach serves as the starting point for all subsequent investigations described in this thesis.

Chapter 4: Tailoring Polishing Steps for Effective Removal of Polysorbate-Degrading Host Cell Proteins in Antibody Purification

Melanie Maier, Stefan Schneider, Linus Weiss, Simon Fischer, Daniel Lakatos, Joey Studts, Matthias Franzreb

Biotechnology and Bioengineering (2024), doi: 10.1002/bit.28767

The research article presented in Chapter 4 extends these findings into downstream processing. Through high-throughput partitioning screens, design-of-experiments studies and chromatographic validation, the study demonstrates how physicochemical properties of hydrolases and antibodies shape co-elution behavior during polishing steps. The study also shows that successful separation strongly depends on the mAb's own characteristics, and that co-elution plays a larger role in hydrolase persistence than previously assumed.

Chapter 5: Quantitative analysis reveals hitchhiking drives polysorbate hydrolase persistence via host cell protein–antibody interactions

Melanie Maier, Lukas Griesinger, Matthias Franzreb, Simon Kluters

Biotechnology and Bioengineering (2026), doi: 10.1002/bit.70170

The research article presented in Chapter 5 introduces an orthogonal persistence mechanism by quantifying reversible hydrolase-antibody interactions using biolayer interferometry. This study is the first to directly determine affinities between different polysorbate-degrading hydrolases and monoclonal antibodies, providing a mechanistic explanation for hitchhiking during Protein A capture. It complements the findings of Chapter 4 by showing how hydrolases can enter the purification train independently of their physicochemical resemblance to the product.

Chapter 6: Specific polysorbate fingerprints of CHO hydrolases and implications for indirect assays

Melanie Maier, Viktor Gross, Linus Weiss, Simon Kluters, Joey Studts, Matthias Franzreb, Patrick Garidel

Journal of Pharmaceutical Sciences (2025), doi: 10.1016/j.xphs.2025.104126

The research article presented in Chapter 6 shifts the focus from downstream behavior to functional characterization of polysorbate degrading hydrolases. The study demonstrates that polysorbate hydrolases exhibit preferences for different polysorbate species. These preferences were resolved using RP-UPLC-MS at subspecies level and revealed that each enzyme exhibits a characteristic fingerprint. Furthermore, the work highlights how these degradation patterns influence commonly used indirect activity assays, showing that such methods can lead to misinterpretation and should not be relied upon for quantitative conclusions.

Chapter 7: A machine learning approach to identify active polysorbate degrading hydrolases in biopharmaceutical formulations

Melanie Maier, Simon Kluters, Joey Studts, Matthias Franzreb, Patrick Garidel and Viktor Gross

Under review in: Journal of Pharmaceutical Sciences (2026)

The research article presented in Chapter 7 builds on the concept of enzyme-specific degradation fingerprints introduced in Chapter 6. It demonstrates that the patterns resulting from hydrolases preferences for individual polysorbate species are sufficiently unique to serve as reliable identifiers. The study applies supervised machine-learning models to fingerprint data to classify and identify active hydrolases in formulations, with a focus on those contributing most significantly to polysorbate degradation. By evaluating multiple classification algorithms, the work introduces a novel approach for functional enzyme identification and provides a basis for risk-based mitigation strategies in biopharmaceutical development.

3 Illuminating a biologics development challenge: systematic characterization of CHO cell-derived hydrolases identified in monoclonal antibody formulations

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mAbs (2024), doi: 10.1080/19420862.2024.2375798

Abstract

Monoclonal antibody (mAb) formulations and other biological drug products have seen a significant increase in enzymatic polysorbate (PS) degradation that reduced stability and jeopardizes the supply of innovative medicines. PS represents a critical surfactant stabilizing the active pharmaceutical ingredients, that are produced by recombinant Chinese hamster ovary (CHO) cell lines. As the list of potential PS-degrading CHO host cell proteins (HCPs) has grown constantly over the past years, tangible data on industrially relevant HCPs are still scarce. By means of a highly sensitive LC-MS/MS method, we investigated 7 different mAb products, resulting in the identification of 12 potentially PS degrading hydrolases, among them was the strongly PS-degrading lipoprotein lipase (LPL). Using a LPL knockout CHO host cell line, we were able to stably overexpress and purify the remaining candidate hydrolases through orthogonal affinity chromatography methods enabling their detailed functional characterization. Applying a PS degradation assay, we have found 9 – mostly secreted – PS-active hydrolases with varying hydrolytic activity. All active hydrolases showed a serine-histidine-aspartate/glutamate catalytical triad. Further, we subjected the active hydrolases to pH-screenings and revealed a diverse range of activity optima, which can facilitate the identification of residual hydrolases during bioprocess development. Ultimately, we compiled our dataset in a risk matrix identifying PAF-AH, LIPA, PPT1 and LPLA2 as highly critical hydrolases based on their cellular expression, detection in purified antibodies, active secretion and PS degradation activity. With this work, we are paving the way towards a comprehensive functional characterization of PS-degrading hydrolases and thus provide a basis for a future reduction of PS degradation in biopharmaceutical drug products.

Keywords: *polysorbate, hydrolase, hydrolysis, degradation, host cell protein, lipase, particle*

3.1 Introduction

Enzymatic polysorbate (PS) degradation in biological drug formulations mediated by host cell proteins (HCPs) has become a widespread and severe challenge in the biopharmaceutical industry. As an indispensable surfactant, PS is used in drug formulations to prevent biopharmaceutical agents – often monoclonal antibodies – from aggregation and minimizes surface adhesion and air-liquid interfacial stress [48]. PS is a heterogeneous mixture consisting of a hydrophilic polyoxyethylene sorbitan head group that is esterified to one or multiple hydrophobic fatty acids of varying length. Degradation of PS is driven via two independent pathways: Chemical degradation by auto-oxidation or hydrolysis and enzymatic degradation elicited by hydrolytic enzymes [48]. Whereas chemical degradation has been successfully prevented by the use of buffering agents and anti-oxidants [8,99,227], hydrolytic enzymes acting on ester bonds (Enzyme class 3.1) turned out to be the major root cause of PS degradation. These enzymes are secreted in the bioprocess as HCPs originating from the production cell lines derived from Chinese hamster ovary (CHO) cells [43,71,140,240]. Here, a small portion of particularly difficult to remove hydrolytic HCPs persists in the downstream purification process and eventually causes hydrolytic cleavage of PS in drug substance and drug product. The degradation of PS results in the accumulation of free fatty acid aggregates and associated (sub-)visible particle formation. This is critical for product safety and quality, as these aggregates can potentially induce adverse immune responses, reduce product efficacy, or compromise its stability. In recent years, significant effort has been put into the identification and characterization of potential HCPs acting on the ester bonds of PS [65,117,121,127,145,197]. One prominent example is Phospholipase B-Like 2 (PLBL2), which was long considered an active polysorbate degrading HCP. However, contaminated recombinant HCP samples resulted in misinterpretations, emphasizing the importance of a well-designed purification and characterization strategy for recombinant HCPs to avoid misleading data interpretation as a result of persisting impurities [237]. As hydrolytic HCPs were found to cause PS degradation at trace levels, <10 ppm [240], detection of these low abundant enzymes represents a major analytical challenge for the biopharmaceutical community. Therefore, new innovative analytical methods were developed including optimized sample preparation prior to mass spectrometric analysis [65,127,145,197] and activity-based-protein profiling [39,117,121]. To date, advances in HCP detection resulted in a comprehensive list of potentially PS-degrading protein contaminants detected at various stages of the bioprocess. Data regarding their enzymatic activity profiles against PS and an understanding of the impact of the downstream purification processes on these enzymes is still lacking. A less explored but promising approach to address this issue is the recombinant overexpression and simultaneous screening of multiple putative polysorbate degrading HCPs, especially the hydrolase species.

Towards this end, we identified, systematically overexpressed and characterized a selected list of potentially critical CHO cell derived hydrolases, which were previously detected in 7 purified model mAb products via a state-of-the-art mass spectrometry approach. We further employed a transposase-based expression system in combination with a newly

established LPL knockout CHO host cell line to generate stable cell pools expressing the candidate hydrolases in high yields, without contamination of LPL and by entailing Strep-tavidin (Strep)- and Histidin (His)-affinity tags. The latter enabled the implementation of orthogonal purification steps, ensuring a considerably high purity of the recombinantly expressed CHO hydrolases. Characterization studies with the purified hydrolases were conducted encompassing cellular localization data, activity testing towards PS degradation and pH activity profiling. Finally, by compiling all data, we were able to build a knowledge base that assists in the prioritization and risk assessment of critical CHO cell derived hydrolases at different points throughout the entire biopharmaceutical production process.

3.2 Materials and Methods

3.2.1 Hydrolase identification by LC/MS-MS

Sample preparation

For each mAb, 8 mg of Ultrafiltration/Diafiltration material was diluted using purified water to the final volume of 370 μ l, and pH was adjusted using 10 μ L of 1 M Tris/ HCl buffer (pH 8.0). 4 μ g of trypsin was added to each sample, and samples were incubated at 37 °C for 2 h, at 550 rpm. Dithiothreitol was added to each sample to 13.4 mM final concentration, followed by 10 min incubation at 95 °C for 10 min, 550 rpm. Undigested mAb was pelleted by 5 min centrifugation at 15000 g, and supernatants were transferred to fresh tubes. Samples were acidified using 15 μ L of 10% formic acid and desalted using C18 StageTips and dried by vacuum centrifuge. Purified dried peptides were fractionated using tip-based basic reversed-phase (BRP) fractionation. For BRP-fractionation 10 mM ammonium formate elution buffers were prepared with ascending CAN concentration. The pH of the elution buffers was adjusted to 10 using 28% NH₄OH and BRP tip columns were prepared using 14 layers of C18 Empore Extraction material (3M). Columns were equilibrated by applying 100 μ l of MeOH, 100 μ l of 10 mM ammonium formate, 20% CAN, and 100 μ l of 10 mM ammonium formate, 5% CAN. Dried samples were resolubilized in 200 μ l of 10 mM ammonium formate, 5% CAN and were loaded to columns, and flow-through was collected as the first fraction after 3 min centrifugation at 2500 g. Subsequent fractions were collected using the elution buffers with 7.5%, 10.0%, 12.5%, 15.0%, 17.5%, 20.0%, and 50.0% CAN, and all fractions were dried using a vacuum centrifuge until further analysis.

LC-MS/MS Data Acquisition

The dried samples were reconstituted in a solution containing 5% acetonitrile (MeCN) and 5% formic acid (FA), and then loaded onto a PepMap® RSLC C18 Easy-spray column (#ES903, ThermoFisher) at a flow rate of 300 nL/min using solvent A (0.1% FA in water) at a column temperature of 60 °C, using a Dionex UltiMate 3000 RSLCnano system (ThermoFisher Scientific, Waltham). Peptides were separated with a 240-minute linear gradient, starting from 98% solvent A and 2% solvent B (0.1% FA in MeCN) to 65% solvent A and

35% solvent B, at a flow rate of 250 nL/min. The column was washed with four cycles of zigzag washing steps, ranging from 2% solvent B to 95% solvent B.

The eluted peptides from the analytical column were subjected to positive ionization at 2.0 kV using the EASY-SprayTM Source (ThermoFisher Scientific, Waltham) of an Exploris 480 mass spectrometer, equipped with a FAIMS pro interface (ThermoFisher Scientific, Waltham). The mass spectrometer was operated in DDA mode with survey scans acquired from m/z 300 to 1600 in the Orbitrap analyzer at a resolution of 60000 at four different compensation voltages (CV -40, -65, -75, -90), followed by fragmentation of the most abundant ions. MS/MS spectra were obtained using higher-energy collisional dissociation (HCD) at 30%. The isolation window was set at 2 m/z , the orbitrap resolution at 15000, the target value at 1E5, and the maximum injection time set at auto. Selected precursor ions for fragmentation (including charge state 2-8) were excluded for 30 s, and the repeat count was set at 1.

Data Analysis

Acquired raw files were analyzed using Proteome Discoverer 2.2 with the Mascot and SEQUEST algorithms in one combined search. The search was performed against the Chinese hamster UniProt proteome database (UP000001075). The applied search parameters were as follows: a full tryptic digestion and a maximum missed cleavage of 3, dynamic modifications of oxidation at methionine and acetylation at protein N-termini, the minimum peptide length of 6, and a maximum of 144. The precursor mass tolerance and fragment mass tolerance were set to 10 ppm and 0.02 Da, respectively. Data filter criteria were set as 1% and 5% FDR for peptides and proteins, respectively. A protein was considered as identified when at least two unique peptides fulfilling the mentioned criteria could be identified. Uniprot [36] and BRENDA [28] databases were used for the enzyme class determination of the HCPs.

3.2.2 Hydrolase quantification by LC/MS-MS

Sample preparation

An equivalent of 100 μ g antibody from harvested cell culture supernatant were denaturated with 80 μ l of 7 M guanidine $\cdot$ HCl, 0.4 M Tris-HCl buffer at a final volume of 180 μ l. Samples were reduced in 5mM DTT for 30 min at 56 °C for 30 min and alkylated with 10mM Chloroacetamide at room temperature in the dark for 45 min. Afterwards, samples were diluted to a final volume of 800 μ l with 25 mM Tris-HCl before adding 1.5 μ g of Trypsin/LysC mix (#V5073, Promega). Digestion was carried out overnight for at least 18h. Digested samples were desalted using PierceTM Peptide Desalting Spin Columns (#89851, ThermoFisher Scientific) as described in the manufacturer's manual and dried until further use.

LC-MS/MS Data Acquisition

The dried samples were reconstituted in 2% acetonitrile (MeCN) and 0.1% formic acid (FA) and trapped on a PepMapTM Neo 5 μ m C18 300 μ m x 5 mm Trap cartridge (#174500,

ThermoFisher Scientific) at a flow rate of 30 μ l/min using a Dionex UltiMate 3000 RSLC-nano System (ThermoFisher Scientific, Waltham). Subsequently, peptides were eluted in back flush on to a PepMap® RSLC C18 Easy-spray column (#ES75500PN, ThermoFisher Scientific) (75 mm inner diameter x 50 cm, 2 mm particle size, 100 Å) with a 120-minute linear gradient at a flow rate of 300 nL/min, starting from 98% solvent A and 2% solvent B (0.1% FA in MeCN) to 65% solvent A and 35% solvent B, at a flow rate of 300 nL/min. Afterwards the column was washed with 80% solvent B before being equilibrated with 98% solvent A and 2% solvent B.

The eluted peptides were subjected to ionization at 2.0 kV using an EASY-Spray™ Source (ThermoFisher Scientific, Waltham) of an Exploris 480 mass spectrometer, equipped with a FAIMS pro interface (ThermoFisher Scientific, Waltham). The mass spectrometer was operated in data dependent acquisition (DDA) mode with full scans acquired from m/z 300 to 1600 in the orbitrap analyzed at a resolution of 60,000 followed by fragmentation of the 10 most abundant ions at three different compensation voltages (CV -50, -65, -85). Tandem mass spectra were obtained using higher-energy collisional dissociation at 30%. The isolation window was set at 2 m/z , the Orbitrap resolution at 15,000, the target value at 1E5, and the maximum injection time set at auto. Selected precursor ions for fragmentation (including charge state 2–8) were excluded for 45s, and the repeat count was set at 1.

Data Analysis

Raw files were analyzed using Proteome Discoverer 3.0 using Mascot and SEQUEST search algorithms in one combined search using the Chinese Hamster TrEMBL proteome database (downloaded 01.03.2023, 57854 entries). Database search was carried out using search parameters as follows: full tryptic digestion and a maximum missed cleavage of 2, dynamic modifications of oxidation at methionine and acetylation at protein N-termini, the minimum peptide length of 6, and a maximum of 144. The precursor mass tolerance and fragment mass tolerance were set to 10 ppm and 0.02 Da, respectively. Data filter criteria were set as 1% false discovery rate (FDR) for peptides and proteins. Peak areas deriving from the full scans were used for relative quantification using the precursor ions quantifier node with default settings in Proteome Discoverer.

3.2.3 Cell culture

CHO K1 producer cell lines were cultured with Boehringer Ingelheim (BI) proprietary medium in 125 mL shake flasks on an orbital shaker (Infors HT, Bottmingen) with 120 rpm (50-mm orbit). Cells were seeded with 5×10^5 viable cells/mL and passaged every 2–3 days. Viable cell density (VCD) and viability were determined by trypan blue exclusion using Vi-CELL BLU (Beckman Coulter, Brea). Cultured cells were maintained at 36.5 °C and 5 % CO₂.

3.2.4 Gene expression analysis

RNA was isolated from 5x10⁶ cells using QIAasympphony RNA Kit and the QIAasympphony (Qiagen, Hilden). The RNA quality was assessed using the Fragment Analyzer with the RNA Kit (DNF-471-0500, Agilent) and samples with RNA quality numbers of ≥ 7.5 were processed further. Strand specific RNA libraries were prepared using the NEBNext Ultra II Directional RNA Library Kit and the NEBNext Poly(A) mRNA Magnetic Isolation Module (#E7760, # E7490, New England Biolabs). Libraries were sequenced on Illumina's NovaSeq 6000 (Illumina, San Diego) in 100 bp paired-end mode generated mean read counts of 3×10^7 read pairs per sample. Sequencing data was analyzed with GeneData Selector® software (Genedata, Basel). Sequence reads were quality filtered, adapter sequences were trimmed off and high-quality reads were mapped to BI's proprietary CHO K1 genome. Protein coding sequences were annotated, counted and normalized to library size. Gene expression was calculated as transcripts per million reads (TPM).

3.2.5 Generation of LPL KO cell lines

Generation of LPL KO cell lines producing mAb was done via CRISPR/Cas9 deletion technology. In brief, 180 pmol Alt-R™ Sp. Cas9 (#1081059, Integrated DNA Technologies) was complexed to 220 pmol Alt-R™ sgRNAs (Integrated DNA Technologies) (Upstream: TCTACCGCGCTCCAGCCCTT; Downstream: GGTCTTGGCGCACTCTAGAA) and transfected via the NEON electroporation system (Thermo Fisher Scientific, Waltham) in 5x10⁶ cells. Transfected cells were resuspended in 25 mL culture medium in 125 mL shaking flasks and single cell sorted via FACSARIA™ (BD Bioscience, San Jose) after 3 days cultivation. Following expansion, biallelic LPL KO clones were identified via (Non-)Deletion PCR of extracted gDNA (Non-Deletion primer:fw: tggggcaacggtactgtaga; rev: aacgtacctgtctgtcg; Deletion primer:fw: tggggcaacggtactgtaga; rev: ctggatgtgaggaagctacttc). In addition, lack of LPL expression on protein level was confirmed by ELISA measurement (#SEA386Mu, Cloud-Clone Corp.).

Deletion of LPL in CHO host cells was performed with a ZFN pair targeting exon 7 (Sigma-Aldrich). 2 µg of each ZFN were transfected with the NEON electroporation device in 1x10⁶ cells and transferred into 10 mL medium in a 50 mL TPP. Single cell cloning was performed 3 days after transfection and LPL KO validation was executed via ELISA.

3.2.6 Fed-batch cultivation

Fed-batch cultivation of LPL KO and LPL wt were performed in ambr15®/ambr250® (Sartorius, Göttingen) micro bioreactors under BI standard cultivation conditions. After 14 days cultivation, supernatant was harvested by centrifugation and filtration.

3.2.7 Generation of stably expressing hydrolase cell lines and recombinant hydrolase expression

For hydrolase expression, a transposase system was used in combination with a glutamine selection system. Hydrolase sequences were extracted from the proprietary CHO K1 transcriptome and cloned into a transposase recognition sequence harboring expression plasmid. For purification purposes, a C-terminal His- and Strep Tag were added to the native hydrolase coding sequences. 3 μ g transposon plasmid and 1.5 μ g transposase expression plasmid were transfected (NEON electroporation system) into 5×10^6 cells and cells were resuspended in 5 mL medium and incubated statically. Selection pressure was applied one day post-transfection and cells were expanded in 40 mL medium. After reaching a viability of >40% and a VCC of $>1.5 \times 10^5$ cells/mL, cells were transferred in 25 mL medium in a shaking flask and cultivated until reaching viabilities >95%. Stable hydrolase expressing cell pools were (fed-)batch cultivated in 1.5 L cultures over 5-12 days and cell pellet/supernatant was harvested via centrifugation and stored at -70°C .

3.2.8 Enzyme localization

Expression and localization of the protein of interest was analyzed with biolayer interferometry using Anti-His2 biosensors (Octet® Anti-HIS2 #18-5114, Sartorius) on an Octet HTX platform (ForteBio, Fremont, CA). For protein localization, cell pellets were lysed using the same volume of lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 1 % Triton X-100) as the corresponding cell culture supernatant. This ensured a consistent comparison between intracellular and extracellular protein levels. The ratio of the amount of intracellular- and extracellular protein was then calculated.

3.2.9 Purification of recombinant hydrolases

Recombinant hydrolases were purified using two orthogonal capture steps followed by size exclusion chromatography on an Akta Avant 150 system (Cytiva, Marlborough). Initially, immobilized metal affinity chromatography (IMAC) was performed using a nickel-nitrotri-acetic acid column (HisTrap FF crude #17528601, Cytiva). The column was equilibrated with buffer containing 20 mM Hepes pH 8, 150 mM NaCl and 20 mM imidazole. After sample loading, the column was washed with the same buffer until baseline absorbance at 280 nm. The bound protein was eluted by an imidazole gradient over 10 column volumes (20-500 mM). Elution fractions were pooled and loaded onto a Strep-Tactin column (StrepTrap XT #29401323, Cytiva) according to manufacturer's instructions. Proteins were eluted with a step elution of 10 mM HEPES, 150 mM NaCl and 50 mM biotin at pH 8. Samples containing the protein of interest, as determined by SDS-PAGE, were pooled and polished by size exclusion chromatography (HiLoad 16/600 Superdex 200 pg #28989335, Cytiva). The recombinant hydrolase monomers were identified by comparing the chromatogram to a molecular weight standard. Fractions containing pure protein were aliquoted and stored at -70°C .

3.2.10 Fluorescence Micelle Assay for determining polysorbate degradation

The assay was performed as described before [216]. Purified hydrolases were spiked into a typical formulation buffer (50 mM Histidine pH 6) at a final concentration of 25 $\mu\text{g/mL}$ and mixture was incubated at room temperature. At different time points (d0, d1, d3, d7, d14), samples were frozen at $-70\text{ }^{\circ}\text{C}$. Hydrolase containing cell culture supernatant was diluted in PS spiked (0.4 mg/mL), fresh cultivation media.

3.2.11 pH dependent activity

The esterase activity assay was fully automated using a Fluent Control (Tecan Group, Männerdorf) and employed 4-methylumbelliferyl decanoate (MUD4) as a substrate to evaluate hydrolytic activity at various pH values between 4 and 8. Multi-component buffers were prepared at pH 4 and 8, and a master mix containing the MUD4 substrate was created. The multi-component buffers were mixed in a 12-column reservoir trough to generate a linear pH gradient. The assay was performed in a 96-well plate with the following composition per well: 75 μL sample, 75 μL water, 150 μL master mix at various pH values. MUD4 is a nonfluorescent ester that, upon cleavage by esterases, releases the fluorescent product 4-methylumbelliferon (MU4). This cleavage of the ester bond results in an increase in fluorescence signal within the sample, which can be detected using excitation and emission wavelengths of 355 nm and 460 nm (Spectra Max M series, Molecular Devices, San José), respectively. By measuring the assay at different pH values, pH-dependent activities can be investigated. The fluorescence intensity is proportional to the amount of fluorogenic substrate (MUD4) being cleaved, allowing for the quantification of esterase activity in the sample under various pH conditions.

3.3 Results

3.3.1 Identification of hydrolytic Host Cell Proteins in formulated mAb products

By means of a highly sensitive liquid chromatography-mass spectrometry (LC-MS/MS) technique (down to 0.3 ppm), 7 mAb products were analyzed for their residual hydrolase content. Of note, the analyzed mAb products were industrially relevant molecules that were purified through a chemistry, manufacturing, and controls (CMC) bioprocess and were formulated to simulate preparation for clinical application. Across all investigated products, 12 hydrolytic enzymes acting on ester bonds (enzyme class, EC 3.1) were identified (Table 3.1).

Table 3.1: List of hydrolytic host cell proteins of the enzyme class 3.1, identified at least once in LC/MS-MS analyses of 7 different antibody formulations.

Uniprot Accession No.	Protein name	EC number	Gene name	Number of prod- ucts showing HCP hits
G3I8P7	GNS (N-acetylglucosamine-6-sulfatase)	3.1.6.14	<i>Gns</i>	6 of 7
G3H6V7	LPL (Lipoprotein lipase)	3.1.1.34	<i>Lpl</i>	6 of 7
G3IMH4	SMPD1 (Sphingomyelinphosphodies- terase)	3.1.4.12	<i>Smpd1</i>	5 of 7
G3HQP6	LIPA (Lysosomal acid lipase)	3.1.1.13	<i>Lipa</i>	3 of 7
G3HN89	PPT1 (Palmitoyl-proteinthioester- ase 1)	3.1.2.22	<i>Ppt1</i>	3 of 7
G3HKV9	LPLA2 (Group XV phospho- lipase A2)	3.1.1.32	<i>Pla2g15</i>	2 of 7
A0A061IFE2	CES1 (Liver carboxylesterase 1-like protein)	3.1.1.-	<i>Ces1</i>	1 of 7
A0A061I7X9	CES1F (Liver carboxylesterase B-1- like protein)		<i>Ces1f</i>	
G3IIG1	CES2C (Carboxylic ester hydrolase 2C)	3.1.1.-	<i>Ces2c</i>	1 of 7
G3IHH9	IAH1 (Isoamyl acetate-hydrolyzing esterase 1-like)	3.1.1.112	<i>Iah1</i>	1 of 7
A0A061IKF8	PAF-AH (1-alkyl-2-acetyl-glycer- ophosphocholine esterase)	3.1.1.47	<i>Pla2g7</i>	1 of 7
A0A061HXX9	PLD3 (Phospholipase D3)	3.1.4.4	<i>Pld3</i>	1 of 7

Although all mAb products were expressed using the same CHO expression platform the number of identified hydrolases in the purified antibody products varied strongly from 1 to 6 out of 7 investigated samples. LPL, GNS, and SMPD1 were frequently identified as hydrolases, but others (CES2C, CES1(F), PLD3, IAH1, PAF-AH) were only found in single products.

To investigate whether the expression of identified hydrolases correlated with their frequency of occurrence in purified antibodies, we analyzed previously compiled transcriptomics and proteomics data sets of 3 different, representative cell line development campaigns (Figure 3.1). Overall, a high and statistically significant correlation of transcriptome and proteome data was observed for all 3 mAb datasets (Figure A1). Strikingly, for LPL, a frequently mentioned hydrolase in the context of PS degradation [32,65,117,121], the high occurrence in many mAb products correlated with high mRNA and protein expression in all investigated production cell lines. This was also true for SMPD1 and LIPA, although here high expression values correlated only with identification in 5 and 3 out of 7 analyzed mAb products, respectively. The hydrolases CES1F and PAF-AH showed high gene and protein expression in one of the investigated mAb formulations. The other identified hydrolases were expressed at much lower levels and occurrence of identification was low, except for GNS, which was identified in 6 out of 7 products (Figure 3.1). In conclusion, expression of hydrolases varies substantially in CHO cells and mRNA and protein expression levels are not sufficiently predictive of the occurrence and abundance of the hydrolase in mAb products, which is probably due to unique interactions of difficult-to-remove hydrolases with the product and/or the chromatography columns.

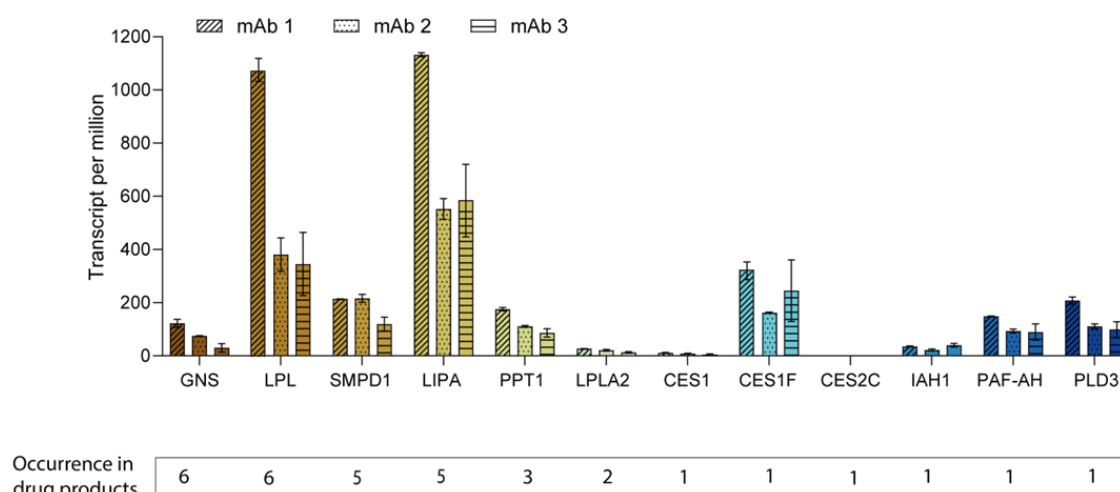
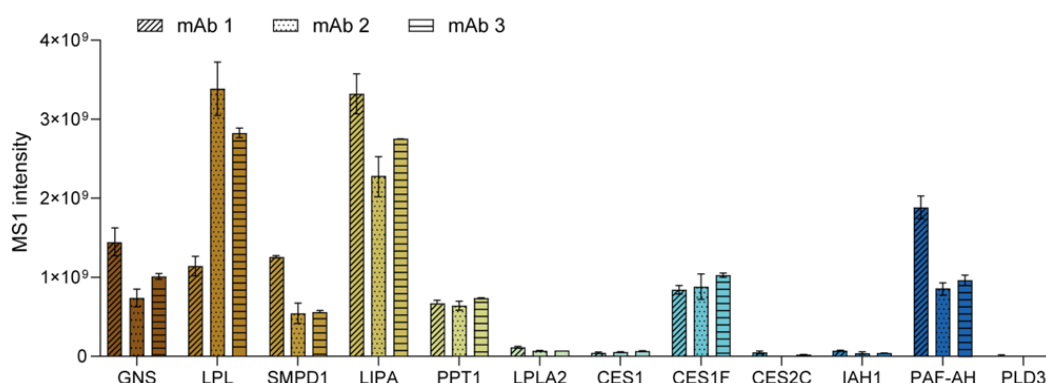
A**B**

Figure 3.1 A: Gene expression data in transcripts per million of potentially PS-degrading hydrolases identified in drug products. Transcript data of producer cell lines from three

different campaigns are depicted. One clone each (two timepoints, $n=2$) was analyzed for monoclonal antibody (mAb) 1 and 2 and a producer pool (one timepoint, $n=24$) was analyzed for mAb 3. Data are sorted in descending order according to their occurrence in 7 investigated drug products. B: Relative protein abundance (MS1 intensity) of potentially PS-degrading hydrolases identified in drug products. Protein expression data in cell culture supernatant from three different campaigns are depicted. One clone each (technical triplicates, $n=3$) was analyzed for mAb 1 and 2, and a producer pool (technical duplicates, $n=2$) was analyzed for mAb 3.

3.3.2 Knockout of PS degrading lipoprotein lipase (LPL) in CHO cells to allow for unbiased HCP characterizations

As LPL was most frequently identified in mAb product formulations, expressed at the highest level in CHO production cells and previously suggested as a source for substantial PS degradation[32], we aimed to investigate the extent of LPL-related PS degradation in an example production process. Therefore, LPL was knocked out (KO) in a mAb-producing CHO cell line using CRISPR/Cas9 genome editing technology. A combination of two different polymerase chain reaction (PCR) experiments validated the complete bi-allelic removal of the LPL gene in the engineered production cell line (Figure 3.2 A). The KO production cell line was fed-batch cultivated head-to-head with the respective originator mAb production cell line (expressing LPL) over a period of 14 days in an ambr@250 bioreactor system. Supernatant sampled on day three of the fed-batch process was measured using LPL enzyme-linked immunosorbent assay (ELISA), which confirmed the absence of LPL protein in samples of the LPL KO cell line (Figure 3.2 B). Next, supernatant harvested at day 14 of the cultivation process was subjected to a fluorescence micelle assay (FMA) measuring PS20 degradation over time at five different time points post PS spiking (d0, d1, d3, d7, d14) (Figure 3.2 C). Strikingly, in the supernatant of the non-edited parental CHO production clone (LPL wildtype (wt)) 95% of initially spiked PS content was degraded already after one day of incubation. In contrast, supernatant of LPL KO production cells elicited a significantly slower PS degradation rate resulting in only 7% degradation of initial PS levels after one day of incubation and 31% degradation after 14 days. While these data suggest a major PS degrading activity by LPL, the observed residual PS degrading activity clearly points in the direction of further hydrolases to contribute to the total PS degradation activity. Therefore, an LPL KO cell line would certainly serve as an improved manufacturing host cell line for biologics but also represents an ideal host cell line to characterize the additionally present hydrolases without the substantially masking PS degradation activity of LPL.

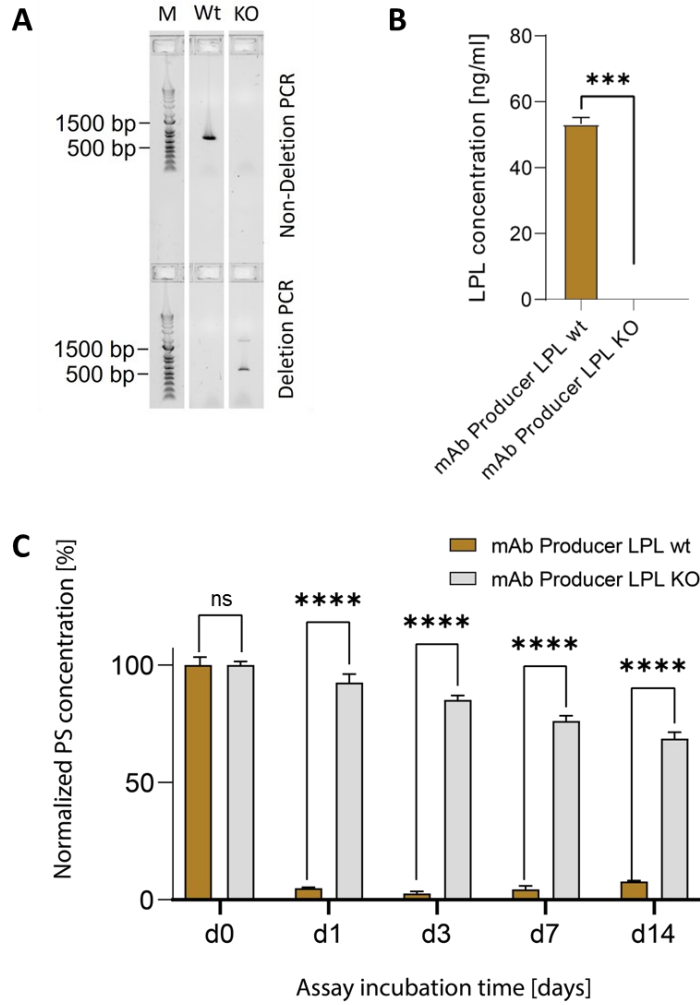


Figure 3.2: A: Agarose gel electrophoresis for verification of biallelic lipoprotein lipase (LPL) knockout (KO) in mAb producer LPL KO (KO) versus mAb producer LPL wildtype (wt). Deletion PCR and non-deletion PCR were performed with genomic DNA. Lack of bands in non-deletion PCRs indicates biallelic deletion. M = Marker. B: LPL concentration in day 3 fed-batch samples of mAb producer cell lines with and without LPL KO. Cell lines were cultured head-to-head in an ambr®250 system and harvested cell culture supernatant was subjected to an LPL ELISA. C: Polysorbate degradation in fed-batch samples of mAb producer cell lines with (brown) and without (gray) LPL assayed over a 14 days time frame. Cell lines were cultured head-to-head over 14 days in an ambr®250 system and harvested cell culture supernatant was subjected to a fluorescence micelle assay. Statistical significance was tested using ordinary two-way ANOVA with Šidák's Multiple Comparison Test. $p < 0.05$ was considered as statistically significant. ns = not significant.

3.3.3 Secretion analysis of CHO hydrolases

In order to characterize the additional hydrolases previously identified in mAb products and to analyze their potential PS degradation activity (Table 3.1), the remaining 11 CHO hydrolases were individually overexpressed in a CHO K1 glutamine synthetase (GS) LPL KO host cell line to avoid the masking potential of LPL. Here, the KO was realized via

zink finger nucleases (ZFN) and validated using LPL ELISA (Figure 3.3 A). Native CHO coding sequences of the 11 hydrolases, containing no additional signal sequence, were cloned into a transposon expression plasmid including His- and Strep-Tags at the 3'-end of the hydrolase gene (Figure 3.3 B). Next, transposon plasmids were stably introduced in the CHO K1 GS LPL KO host cell line using a transposase technology in combination with a GS based metabolic selection approach to generate stable hydrolase expressing pools. As many of the identified hydrolases were annotated by UniProt as intracellular proteins, the question was raised why the potentially intracellular hydrolases were identified in the harvested supernatant and ultimately in the formulated mAb products. To determine whether the hydrolases are actively secreted into the supernatant, their localization in supernatant and cell pellet was quantified via the His-Tag after a three-day batch cultivation of all generated hydrolase overexpressing cell pools that displayed > 98% viable cells at harvest (Figure A2). While the hydrolases PAF-AH and SMPD1 were fully secreted by the CHO cells, other proteins including CES1F, CES2C, GNS, LPLA2, and PPT1 were partially detected in the intracellular fraction. Only LIPA, IAH1, and PLD3 were mainly localized intracellularly (Figure 3.3 C). Other than PLD3, LIPA and IAH1 could be quantified in supernatant of the 3 mAb producer cell lines (Figure 3.1B), which might be due to mAb-HCP shuttling mechanisms or simply a reduced harvest viability of the assayed cell culture (59, 84, 88% for mAb1, 2, 3, respectively). As most of the overexpressed native CHO hydrolases are actively secreted, their occurrence in mAb products is not surprising.

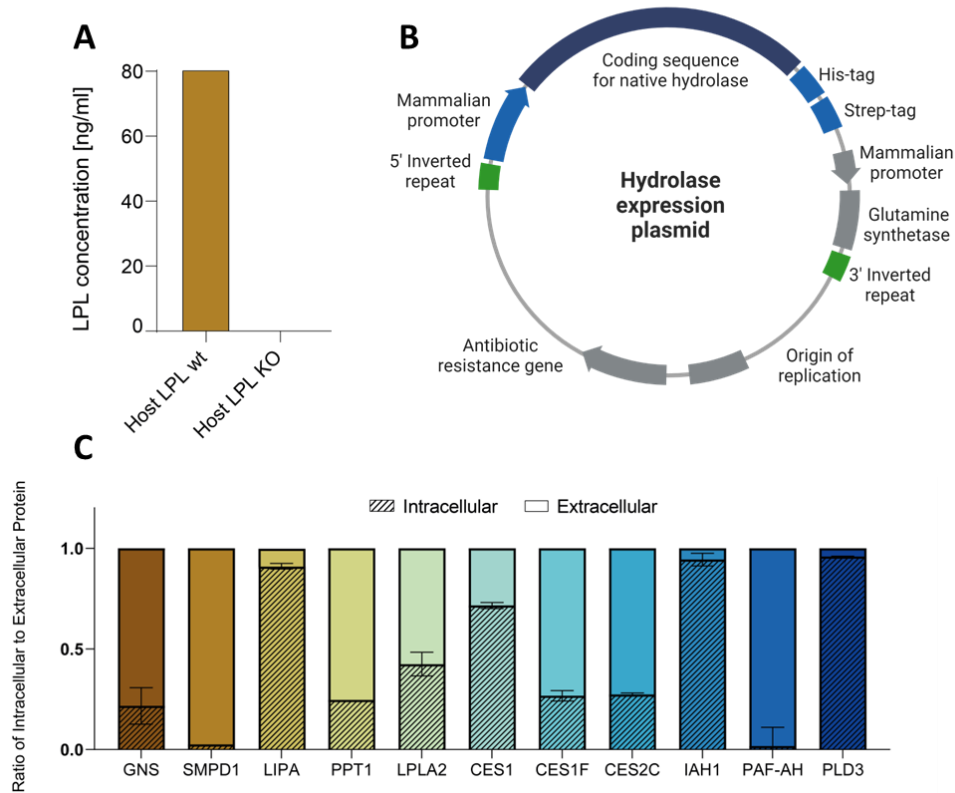


Figure 3.3: A: LPL concentration in day 3 fed-batch samples of mAb host cell lines with and without LPL KO. Cell lines were cultured head-to-head in an ambr®15 system and harvested cell culture supernatant was subjected to an LPL ELISA. B: Hydrolase expression plasmid suitable for transposase-mediated stable transfection via inverted repeats recognition sites. His- and Strep- tags were added at the 3'-end of the sequence coding for the native hydrolase. Glutamine synthetase was used for stable selection. Created with BioRender. C: Relative protein amounts of expressed hydrolases as the ratio of intracellular to extracellular protein. Hydrolases were quantified using biolayer interferometry with his2 sensors on the Octet system. Average values of two replicates with standard deviations are shown.

3.3.4 Hydrolytic activity of CHO hydrolases against polysorbate

Next, we aimed for a detailed characterization of all overexpressed CHO hydrolases regarding their PS degradation potential. All stable hydrolase-expressing cell pools were cultivated in (fed-) batch mode and supernatants were harvested (Figure A2). The crude supernatant was spiked with PS (0.4 mg/mL) and PS degradation was investigated over a period of 14 days via FMA. Strong PS degradation activity was measured for hydrolases CES1F, CES2C, PPT1, and PAF-AH (Figure 3.4 A). Furthermore, moderate PS degradation was elicited by LPLA2, and IAH1, while no PS degradation was observed in

supernatants of cell pools overexpressing GNS, SMPD1, LIPA, CES1 and PLD3, which also served as a comparative baseline. Notably, the crude culture supernatant does not allow for the determination of the exact PS degradation activity for each enzyme, as the supernatant likely contained additional or lacked hydrolases that are not efficiently secreted. Thus, for a direct comparison with uniform starting concentrations we developed a multi-step purification strategy to obtain highly purified protein. Towards this end, over-expressed proteins were purified from harvested cell culture supernatant or cell pellets using a combination of two orthogonal capture steps including an immobilized metal ion affinity column (IMAC) specifically binding the His-Tag on the recombinant hydrolases followed by a Strep-Tactin column to further capture the Strep-tagged hydrolases. In addition, size exclusion chromatography was performed as a polishing step and for aggregate removal. After purification, the CHO hydrolases were tested for their catalytic activity towards PS20 via FMA. Contrary to the FMA performed with crude supernatant, purified enzymes were spiked at uniform concentrations in a formulation buffer containing PS20 and PS degradation was investigated over a period of 14 days as described before (Figure 3.4 B). In contrast to the previous analysis of crude cell culture supernatant, purified CES1, IAH1 and LIPA now showed strong PS degradation activity. This observation aligns with the fact that these enzymes are not secreted and, as a result, are not present in the crude supernatant (see Figure 3.3 C). Strikingly, LPLA2 and PAF-AH now displayed the strongest PS degradation activity among all assayed hydrolases. For CES1F and CES2C, similar PS degradation was observed, while the hydrolase PPT1 now exhibited lower PS degradation activity as a purified enzyme. As before, GNS, SMPD1 and PLD3 displayed no PS degradation potential. Besides the above-mentioned drawbacks when measuring crude cell culture supernatant, it is important to note that the stability or aggregation of purified enzymes can influence the observed activity levels over time. In comparison to other hydrolases, PPT1 was particularly prone to aggregation during the purification process (Figure A3).

These experiments showed that hydrolase containing crude cell culture supernatant can provide an initial indication of hydrolase specific PS degradation potential, however, purification of specific hydrolases is necessary to determine their specific enzymatic activity. In summary, 8 out of 11 hydrolases previously identified in the mAb formulations displayed significant PS degradation activity. Of note, enzyme activity was measured under uniform pH conditions, whereby individual pH optima of the enzymes were not considered.

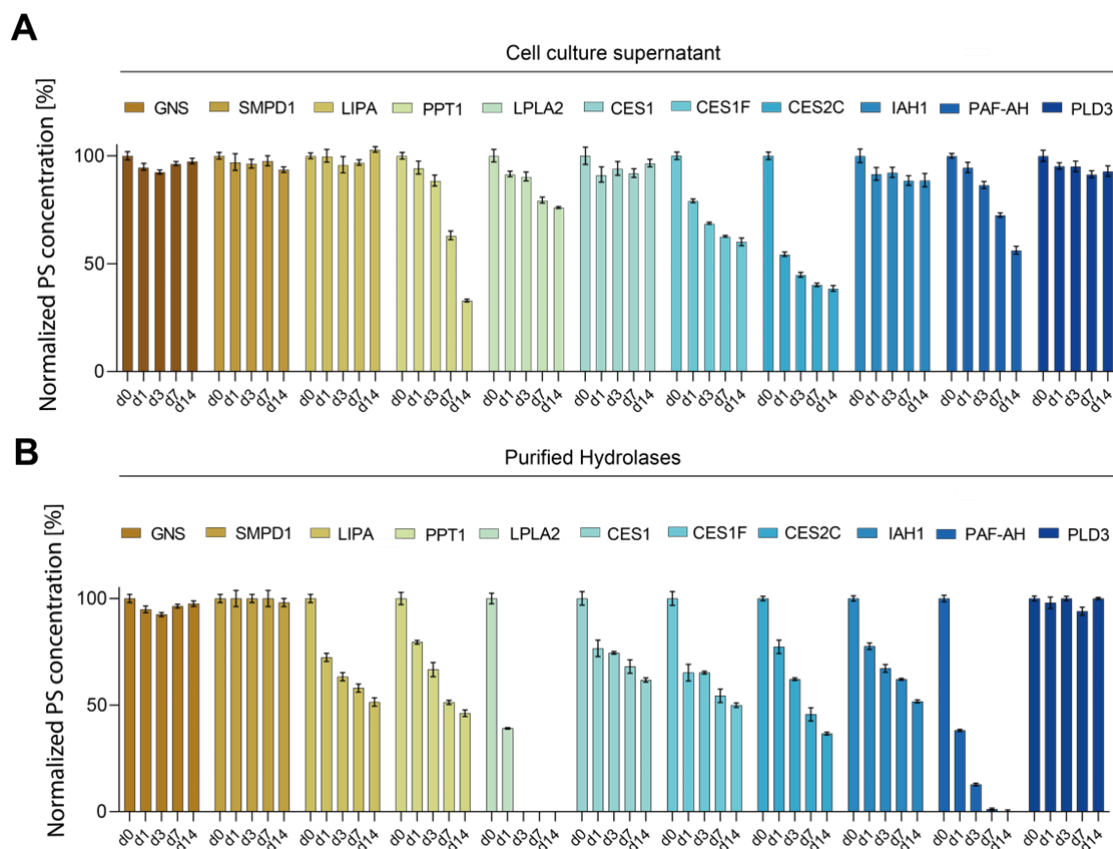


Figure 3.4: A: Polysorbate (PS) degradation over time of crude cell culture supernatant of hydrolase expressing cell lines determined via the fluorescence micelle assay. Cell culture supernatants of (fed-)batch fermentations were diluted in PS-spiked fresh cultivation medium and degradation was monitored over 14 days (d0, d1, d3, d7, d14). PS concentration was normalized to the initial PS concentration on d0. Average values of four technical replicates with standard deviations are shown. B: PS degradation over time of purified hydrolases determined via the fluorescence micelle assay. Purified hydrolases were spiked into a formulation buffer to a final concentration of 20 $\mu\text{g/mL}$ and degradation was monitored over 14 days (d0, d1, d3, d7, d14). PS concentration was normalized to the initial PS concentration on d0. Average values of four technical replicates with standard deviations are shown.

3.3.5 Comparison of active sites in the CHO hydrolases

To identify common features among the identified active hydrolases, we compared the active sites of both PS degrading and non-degrading hydrolases. By referring to UniProt annotations we determined the active site residues of the CHO hydrolases. In addition, we compared the protein sequences to orthologous enzymes with experimentally determined active sites (Table 3.2). A common catalytic triad consisting of a serine (Ser) – histidine (His) – aspartate/glutamate (Asp/Glu) motif was identified in PS degrading hydrolases, which distinguished them from non-PS degrading enzymes (Table 3.2).

Table 3.2: Active site residues of hydrolases identified in 7 industrial mAb formulations. Residues based on indicated literature and UniProt.

Protein	Reference	Active site	Active against PS
CES1	[207]	Ser221, Glu353, His466	Yes
CES1F	[207]	Ser200, Glu332, His442	Yes
CES2C	[176]	Ser228, Glu345, His457	Yes
GNS	[6,134,167]	FGly (from Cys), Asp, His, Arg*	No
IAH1	[126]	Ser71, Glu228, His231	Yes
LIPA	[159]	Ser172, Asp343, His372	Yes
LPL	[51]	Ser159, Asp183, His268	Yes
LPLA2	[63]	Ser198, Asp360, His392	Yes
PAF-AH	[174]	Ser271, Glu295, His350	Yes
PLD3	[146]	His, Asp, Lys*	No
PPT1	[10]	Ser115, Asp233, His289	Yes
SMPD1	[64]	Asp, His, Glu/Asn*	No

*Residue positions are not known

Enzymes with alternative catalytic sites (GNS, PLD3 and SMPD1) did not exhibit PS degradation activity. This finding is particularly valuable when new hydrolases are identified, as it provides a clear marker of potential PS degradation activity. It is important to note that previous research has shown that PS-degrading hydrolases are not limited to the enzyme sub-class of “carboxylic ester hydrolases (3.1.1) [65]. In fact, they can also be found within the broader enzyme class “acting on ester bonds” (3.1), which includes not only carboxylic-ester hydrolases but also thioester and phosphoester hydrolases, among others. This underscores the diverse enzymatic landscape involved in PS degradation and highlights the importance of the conserved catalytic triad as a predictive marker for PS degradation activity.

3.3.6 pH dependent activity of hydrolytic HCPs

The activity optimum of enzymes is strongly dependent on an ideal pH [14]. To further characterize and evaluate the pH optima of each identified hydrolase acting on PS, pH screenings with the purified enzymes were conducted (Figure 3.5 A). Here, enzymatic activity was measured upon cleavage of ester bonds in an assay employing 4-methylumbelliferyl decanoate (MUD4) as a PS surrogate. LIPA and LPLA2 displayed unique activity

profiles with activity optima in a neutral or acidic pH, respectively. In contrast, the group of CES1, CES2C, IAH1 and PAF-AH revealed similar pH dependent activity patterns with low activity in the low pH range and increasing activity with increasing pH values. Equally, the group of CES1F and PPT1 displayed pH optima at strongly basic pH values, while here activity at lower pH was also relatively high.

As the investigated hydrolases showed various pH-dependent activity profiles, this property might be harnessed to identify or exclude residual hydrolases in final drug products. To test this hypothesis, the ester cleaving activity of three model mAb formulations was analysed in the same way as in the above-described pH screenings (Figure 3.5 B). Varying pH-dependent PS degrading activities were observed, which may indicate the presence of various residual hydrolases with unique pH optima. mAb formulation A showed strong activity towards higher (basic) pH values, but no enzymatic activity under acidic pH conditions. In contrast, mAb formulation B displayed strong similarities to the combined activity profiles of LPLA2 and LIPA. Lastly, mAb formulation C also showed strong activity towards higher pH values. However, unlike mAb A, mAb C revealed a medium activity in the acidic pH range, suggesting the presence of CES1F and/or PPT1. In conclusion, differing pH activity profiles might be used to infer the presence or absence of specific hydrolases and could be a valuable resource when considering pH values of mAb formulation buffers.

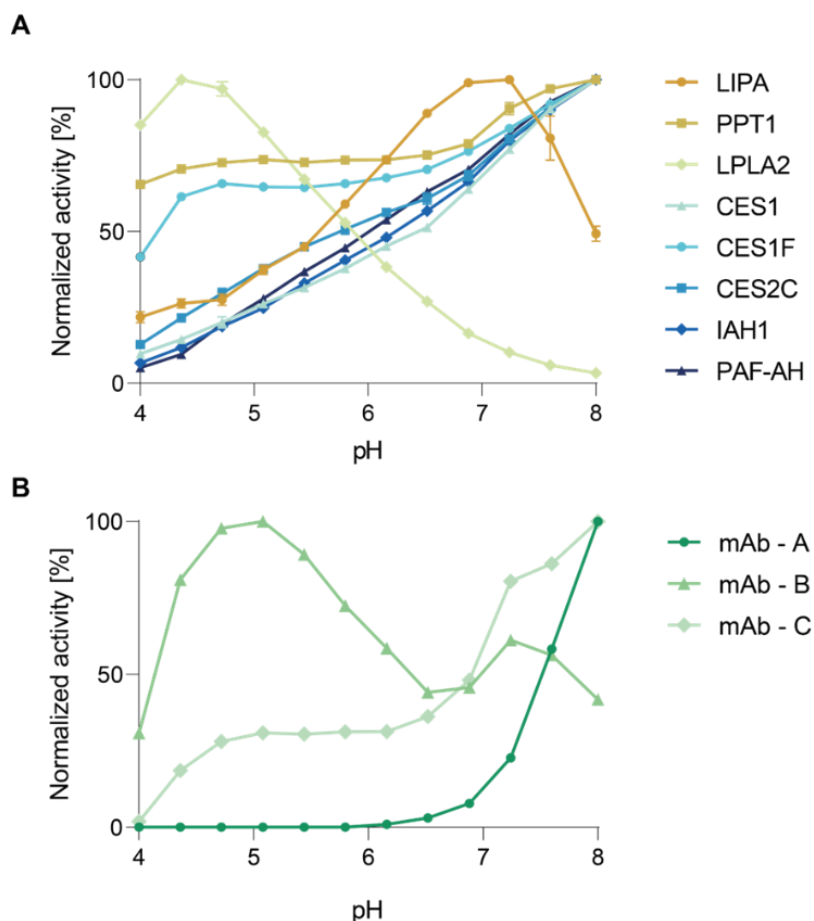


Figure 3.5: A: pH dependent activity of the eight active hydrolases after purification. Values are normalized to highest activity of the respective hydrolase. Average values of duplicate analyses are shown. B: Normalized hydrolytic activities of three mAb samples after the second polishing step at different pH values. Values are normalized to highest activity of the respective mAb sample. Average values of duplicate analyses are shown.

3.3.7 CHO hydrolase risk matrix

In a final step, gathered data about actively PS-degrading hydrolases were summarized in a risk matrix (Figure 3.6). This can serve as a guideline for prioritizing potentially critical HCPs for targeted removal or monitoring during bioprocess development. Based on identified values for gene expression, occurrence in mAb products, cellular localization, and the PS degradation activity, risk points were assigned and ranked in a final score. All risk categories were weighted equally (for rating approach see Table A1). The pH dependent hydrolase activity was not incorporated into the risk matrix, as this factor is specific to the formulation buffer and has to be assessed individually. Of note, proteomic data was not part of the risk matrix, as quantitative proteomic datasets may not be readily available for biopharmaceutical production processes. In any case we were able to demonstrate strong correlation between proteome and transcriptome datasets (supported by localization data). As a result of this assessment, top ranked and thus to be prioritized in removal during the bioprocess are the hydrolases LIPA, PPT1, PAF-AH and LPLA2, which all showed strong PS degradation activity likely catalyzed by the Ser, His, Asp/Glu triad.

Among these, LIPA was the only intracellular hydrolase and was also highly expressed on mRNA level thus rendering this enzyme particularly critical for bioprocesses suffering from low cell viability or high cell lysis at the end of the fermentation and/or during harvest. LPLA2 and PAF-AH both displayed high PS degradation capacity, for PAF-AH in combination with efficient secretion, which rendered it with the higher risk score. IAH1 has the lowest risk score as it combined medium PS activity, intracellular localization and comparably low mRNA expression. This risk matrix can be used as a guideline to focus on the most critical PS degrading HCPs in a CHO based manufacturing process and helps to ensure an efficient utilization of resources.

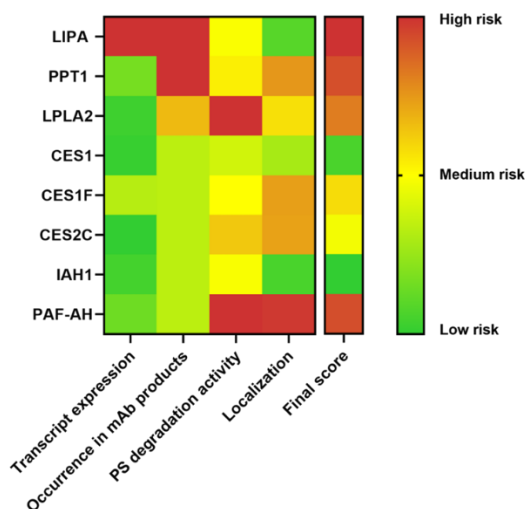


Figure 3.6: Risk matrix summarizing investigated host cell protein (HCP) parameters. Only PS-degrading hydrolases are compared. Color gradient from green to red represents increasing risk of HCP to end up in drug product and/or causing PS degradation. Final score can be used for prioritization of HCP removal.

3.4 Discussion

In recent years, PS hydrolyzing HCPs have posed significant challenges to the biopharmaceutical industry jeopardizing the supply of innovative medicine [43,48,71,140]. Thus, the identification and characterization of hydrolytic HCPs in purified mAb products are crucial for understanding their potential impact on product stability and efficacy and thus safety. In this study, we characterized 12 hydrolytic enzymes potentially acting on PS that were identified in 7 different mAb preparations. Our results suggest that the presence of specific HCPs in purified mAbs is influenced by factors beyond gene expression levels, which is in line with literature on difficult-to-remove HCPs. Here, final formulation “contaminating” HCPs are reported to persist after purification either via product association or co-elution due to product-resembling physicochemical protein properties [2,114,185,187,205].

In line with the previous work of Chiu et al., our study highlighted the importance of LPL as a major contributor to PS degradation [32]. Nevertheless, a CRISPR/Cas9 mediated

LPL KO alone was not able to halt PS degradation completely, explaining the increasing efforts put into identification and characterization of additional hydrolytic HCPs [65,117,121].

By the systematic overexpression of hydrolytic HCPs other than LPL, we aimed for a system enabling detailed characterization and, thus, a comprehensive risk assessment of potentially critical CHO cell derived HCPs acting on PS. We successfully demonstrated that hydrolase containing crude cell culture supernatant can be applied in a PS degradation assay providing strong indications towards hydrolase specific PS degradation. In fact, only LIPA, IAH1 and CES1 did not confirm the results obtained with the purified hydrolases, which is probably due to their intracellular localization.

Following hydrolase purification, FMA data confirmed the list of PS-critical HCPs previously identified by several different groups [32,65,71,106,240]. In our study, we observed that the hydrolase SMPD1 was not active against PS, which presents a discrepancy with the findings reported in literature [70,106]. Kovner et al. demonstrated very weak activity of SMPD1 against PS. However, it is important to consider that this could be attributed to residual HCPs, as only one affinity tag was used to purify hydrolases. In our study, we successfully purified SMPD1 as confirmed by a size exclusion chromatogram and Western blot against the C-terminal His-tag (Figure A4). Furthermore, comparison of the separations of a SEC protein standard mixture and SMPD1 shows that the protein elutes according to the apparent molecular weight of the monomer (72 kDa), demonstrating that no aggregation has occurred (Figure A4). To our knowledge, the present work is the first to include three orthogonal capture steps for hydrolase purifications and activity data based on a uniform starting concentration enabling direct comparison of PS degradation rates. Here, we demonstrate varying enzymatic activity for different hydrolases, which explains why enzyme abundance does not consistently correspond to the extent of PS degradation in a drug product.

Another important aspect is the localization of hydrolytic HCPs, especially in terms of CHO proteins as localization data are oftentimes not experimentally determined. Low harvest viabilities lead to the release of intracellular HCPs changing the HCP profile in the cell culture harvest including PS degrading enzymes.[82] Thus, cell viability at harvest and cell-straining harvest procedures can be of particular importance in products suffering from predominantly intracellular HCPs.

Enzymes active against PS were found to possess the classical catalytic triad, which consists of Serine (Ser), Histidine (His), and Aspartate/Glutamate (Asp/Glu). This finding is consistent with the activities of PS hydrolases investigated in previous research [65,117]. However, acid ceramidase possessing the same catalytic triad does not exhibit PS degrading activity [65,117]. In contrast to the enzymes investigated here, acid ceramidase belongs to EC number 3.5 and not 3.1. Thus, the catalytic triad is a prerequisite for PS degradation but other protein properties are similarly important.

Furthermore, we found that purified mAb formulations exhibited varying activity pH profiles, suggesting the involvement of multiple enzymes in PS degradation. This further

supports the idea that the specific enzymes, which are active with regards to PS degradation, depend on the downstream purification process and the specific monoclonal antibody product. Although these data do not conclusively identify a hydrolase active in a drug product, activity pH profiles can help exclude specific enzyme groups and provide information on which group is significantly involved in PS hydrolysis. This information is particularly useful when multiple hydrolases are identified, and it is unclear which of them are primarily involved in PS hydrolysis in a particular product. Furthermore, it can assist in developing potential preventive strategies such as changing the pH of a formulation buffer to reduce PS-degrading activity of specific HCPs. However, it should be considered that pH changes are limited to the stability range of the product and may cause chemical PS degradation in extreme pH conditions [49,62].

Based on our findings, we developed a risk matrix to prioritize hydrolases for further investigation and potential mitigation strategies. Of note, LPL would most likely have scored the highest score in the risk matrix as it showed highest gene and protein expression values and was found in 6 out of 7 mAb products. In addition, LPL wt cell lines strongly degraded PS in comparison to LPL KO cell lines, which is in line with current literature confirming strong PS degradation by LPL [32]. Strikingly, LIPA, despite being mainly localized intracellularly, is frequently found in mAb preparations and scores the highest final score in the risk matrix. According to our data, approximately 10 % of LIPA is secreted or at least found in the cell culture supernatant. Considering its high expression level, it is conceivable that even at low secretion levels, relatively high concentrations of LIPA are reached in the cell culture supernatant comparable to or even higher than that of other PS hydrolases. Additionally, low harvest viabilities or increased cell lysis induced by the harvest method or necrosis might increase the release of intracellular LIPA, offering an easy-to-implement counter measurement. Although the risk matrix provides a guidance of which hydrolases should be more closely monitored in the final drug product, individual products can differ in their hydrolase profile. Therefore, an individual case assessment is necessary considering also other factors as the pH dependent activity profiles or the level of similarity towards the specific biopharmaceutical.

This work provides a valuable overview facilitating decision-making towards future strategies to mitigate PS degradation. Due to the individual inherent features of the identified HCPs, multiple mitigation strategies are conceivable and must/can be combined to develop a PS-degradation-free final drug product. Starting with the cell line development, it was shown, that critical HCPs can be removed completely using genome engineering tools such as zinc finger nucleases or CRISPR/Cas9 [32,104]. Another cell line engineering approach would be the knockdown of several hydrolases using artificial miRNAs or siRNAs [216]. Given the essentiality of some HCPs for the CHO cell, other methods focused on the two main mechanisms responsible for retaining HCPs during purification: product association or product co-elution [187]. Tackling product association of specific HCPs, the molecule itself could be engineered to prevent specific interactions [75]. However, this concept is only feasible if the functionality of the product remains intact [160]. More common is the use of stringent wash buffers during the downstream chromatography steps, which may include extreme pH values and wash additives [35,119,185]. It is important to note that

not every wash buffer can be used as some may compromise product quality. In general, HCP removal during purification can be realized by many other methods as for example flocculation [93], depth filtration [229], and various chromatography modes [119]. Of note, industrial bioprocesses are striving for platform processes that mostly do not allow the introduction of additional HCP reduction steps to ensure timely development and reduced complexity. Thus, with the help of the presented study it will be possible to optimize and adapt existing purification processes to specifically remove critical HCPs. Finally, PS degradation can be reduced by alternative formulation strategies. Besides changing the pH of the formulation buffer, the addition of hydrolase inhibitors or fatty acid solubilizer were successfully applied to reduce PS degradation and/or particle formation [169]. Moreover, alternative surfactants as Poloxamer 188 were effectively used due to their lack of cleavable ester bonds [169]. However, other than PS20, which has an excellent safety profile, any new formulation ingredient must address toxicology and stability concerns, requiring laborious and time-consuming safety analyses [48,169]. Our system of stably HCP overexpressing CHO cells enabled the robust supply of HCPs without a contamination by the strongly PS degrading LPL enzyme and facilitates the expansion of this dataset in the future. In conclusion, PS degradation represents a highly relevant problem in the biopharmaceutical industry that can only be solved through joint efforts of all bioprocess-involved departments.

Acknowledgements

The authors gratefully acknowledge support by team members of ADB, CLD, LS DSP, ES PD from Boehringer Ingelheim Pharma GmbH & Co. KG.

4 Tailoring Polishing Steps for Effective Removal of Polysorbate-Degrading Host Cell Proteins in Antibody Purification

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Biotechnology and Bioengineering (2024), doi: 10.1002/bit.28767

Abstract

Ensuring the quality and safety of biopharmaceutical products requires the effective separation of monoclonal antibodies (mAbs) from host cell proteins (HCPs). A major challenge in this field is the enzymatic hydrolysis of polysorbates (PS) in drug products. This study addresses this issue by investigating the removal of polysorbate-degrading HCPs during the polishing steps of downstream purification, an area where knowledge about individual HCP behavior is still limited.

We investigated the separation of different mAb formats from four individual polysorbate-degrading hydrolases (CES1F, CES2C, LPLA2 and PAF-AH) using cation exchange (CEX) and mixed-mode chromatography (MMC) polishing steps. Our research identified a key challenge: The similar elution behavior of mAbs and HCPs during chromatographic separation. To investigate this phenomenon, we performed high-throughput binding screenings for recombinant polysorbate-degrading hydrolases and representative mAb candidates on CEX and MMC chromatography resins. We then employed a three-step strategy that also served as a scale-up process, optimizing separation conditions and leading to the successful removal of specific HCPs while maintaining high mAb recovery rates (> 96 %). This strategy involved the use of surface response models and miniature columns for screening, followed by validation on larger columns using a chromatography system.

Our results highlight the critical role of the inherent properties of monoclonal antibodies for successful separation from HCPs. These results underscore the need to tailor the purification process to leverage the slight differences in binding behavior and elution profiles between mAbs and specific HCPs. This approach lays the foundation for developing more effective strategies for overcoming the challenge of enzymatic polysorbate degradation, paving the way for improved quality and safety in biopharmaceutical products.

Keywords: *downstream development, host cell protein, hydrolase, monoclonal antibody, polysorbate*

4.1 Introduction

Monoclonal antibodies (mAbs) are the leading molecule class among biopharmaceuticals with significant therapeutic potential.[90] To enhance protein stability and extend shelf-life, surfactants are applied in mAb formulations to protect against interfacial stresses. Among the surfactants commonly used in biopharmaceutical drug formulations, polysorbates, specifically polysorbate 20 (PS20) and polysorbate 80 (PS80), are prone to degradation and hydrolysis under conditions commonly used in the industry [99,100]. This degradation can compromise the stability and therefore the efficacy of the mAb formulations, leading to potential safety concerns and reduced therapeutic efficacy [102].

The purification of mAbs from host cell proteins (HCPs), especially the removal of polysorbate-degrading HCPs, remains a critical challenge. HCPs are process-related impurities introduced during mAb generation by the mammalian production cell lines and must be reduced to appropriate levels to ensure product stability and safety.

Most mAb purification trains involve an initial affinity chromatography step followed by two polishing steps. The HCPs found in the Protein A eluate are primarily the result of interactions with either the chromatography resin and ligands or the mAbs [114]. Despite a significant reduction in total HCP concentrations in the Protein A elution pools, typically several hundred HCPs are still detected after this unit operation [149]. Since most of the HCPs are more acidic than mAbs, a large fraction of the HCPs can be successfully separated in the subsequent polishing steps [89].

Monitoring HCP levels in the downstream process is crucial, and enzyme-linked immunosorbent assays (ELISAs) are commonly used because of their efficiency and speed. However, they have limitations, such as specificity, sensitivity, and lack of information on individual HCPs. Some studies even suggest that certain polysorbate-degrading hydrolases may remain undetected by commercial HCP ELISAs [70]. As the risks posed by individual HCP species vary, it is essential to implement methods that can provide information on individual HCPs as an orthogonal strategy for risk reduction [19].

While there is existing knowledge on overall HCP content, little is known about the behavior of individual HCPs. The incomplete knowledge of their identities and their extremely low abundance, often less than 1 ppm, has made this task particularly difficult.[118] To address these challenges, significant research efforts have been devoted to the identification, detection, and quantification of HCPs responsible for polysorbate degradation.[91,117,225] These approaches have identified several HCPs involved in polysorbate degradation, including carboxylesterases (CES2C and CES1F) [50,106,240], lipoprotein-associated phospholipase A2 (LPLA2, also known as Pla2g15) [71], platelet-activating factor acetylhydrolase (PAF-AH, also known as Pla2g7) [39,117], palmitoyl-protein thioesterase 1 (PPT1) [65,106], Lipoprotein lipase (LPL) [32], and lysosomal acid lipase (LIPA) [39,236].

Recent studies suggested that ultra-low affinity HCP-mAb interactions may allow polysorbate-degrading hydrolases to evade purification processes [75]. However, there is still a

need for a more comprehensive understanding of the behavior of individual polysorbate degrading HCPs during downstream purification steps, as well as the development of targeted approaches to minimize their levels in the final product.

In this study, we aim to fill this knowledge gap by systematically characterizing the behavior of four polysorbate degrading HCPs during downstream polishing chromatography steps. To achieve this, we first generated these enzymes by recombinant expression in a CHO host cell line, followed by purification via orthogonal steps. Our study employed a three-step strategy involving high-throughput screenings on slurry plates, separation optimization on miniature columns and validation of optimized separation conditions on packed bed columns. This comprehensive approach allowed for the systematic investigation of the binding behavior of individual polysorbate degrading hydrolases and mAbs under various conditions, as well as the identification of optimal separation conditions. By understanding the factors that influence the separation efficiency between mAbs and polysorbate degrading HCPs, we can optimize the purification process to get rid of these culprit enzymes. Using this three-step strategy, the study successfully identified and validated optimal purification conditions for the effective separation of specific polysorbate-degrading HCPs from various mAb formats.

4.2 Material and Methods

4.2.1 Model Antibodies

In this study, several model antibodies were selected based on their differences in molecular format, molecular weight (MW), and isoelectric point (pI) (Table 4.1). These criteria were chosen to cover a wide range of behavior during the polishing steps of the purification process. All model antibodies were produced in-house (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany).

Table 4.1: Characteristics of the five monoclonal antibodies (mAbs) investigated in this study.

Molecule	Classification	Isoelectric Point (pI)	Molecular weight (MW) [kDa]
mAb - 1	IgG1	9.6	145
mAb - 2	IgG4	7.8	146
mAb - 3	IgG4	7.4	144
mAb - 4	Bispecific mAb	9.3	151
mAb - 5	IgG-scFv	8.7	199

4.2.2 Expression and purification of recombinant host cell proteins

Hydrolase expression was achieved using a transposase system and a glutamine selection system, with hydrolase sequences extracted from the CHO K1 transcriptome and cloned into a transposon harboring expression plasmid. Post-transfection, cells were subjected to

selection pressure, expanded, and then transferred to a shaking flask for cultivation. Stable hydrolase-expressing cell pools were cultivated and harvested. For the purification of recombinant hydrolases, a two-step capture process was followed by size exclusion chromatography. The process involved the use of immobilized metal affinity chromatography (IMAC) and a Strep-Tactin column, with the final purified protein aliquoted and stored at -70 °C. Protein concentrations were determined by absorbance at 280 nm using NanoDrop protein quantification (Thermo Fisher Scientific, Waltham, USA).

Table 4.2: Characteristics of the expressed and purified polysorbate hydrolases with abbreviations used and their Uniprot accession numbers.

Polysorbate Hydrolase	Abbreviation	Gene name	pI	MW [kDa]	Uniprot Accession
Carboxylesterase 1f	CES1F	<i>Ces1f</i>	7.9	65	A0A061I7X9
Carboxylic ester hydrolase	CES2C	<i>Ces2c</i>	5.8	65	G3HIG1
Group XV phospholipase A2	LPLA2	<i>Pla2g15</i>	6.3	50	G3HKV9
1-alkyl-2-acetyl-glycerophospho-choline esterase	PAF-AH	<i>Pla2g7</i>	8.6	52	G3HTI7

4.2.3 High-throughput batch binding screenings

We performed K_p (partition coefficient) screenings with hydrolases and monoclonal antibodies (mAbs) to identify optimal separation conditions by calculating separation factors. This approach was inspired by the high-throughput screening method described by Kelly et al. and McDonald et al. [97,98,139].

To determine the K_p for purified hydrolases and mAbs, automated high-throughput batch binding screenings were performed using MultiScreen® 96-well 0.45 µm filter plates, pre-filled with 25 µL of target resin (POROS™ XS or Capto adhere).

A Tecan Fluent automated liquid handling system was used for resin preparation and liquid transfer. The K_p-Screening included two equilibration steps, one loading step, and two stripping phases, each with a 200 µL liquid phase volume. Equilibration and loading were conducted on 48 wells of the resin plate, with specific pH and counter ion concentration combinations, enabling 48 unique load conditions per experiment. For CEX and MMC K_p-Screening, a multi-component buffer system was used according to Kröner et al. [110]. The mAb and HCP stock solutions were exchanged into the corner buffers using a 53 mL HiPrep 26/10 Desalting Column.

The K_p value is the quotient of the protein concentration bound (q_p) to the resin and the protein concentration in the supernatant (c_p) as described by formula (1).

$$K_p = \frac{q_p}{c_p} \quad (1)$$

Using the K_p value of an HCP (impurity) and the mAb (target) the separation factor α was calculated for a given counterion concentration and pH, according to formula (2).

$$\alpha = \frac{K_{p \text{ impurity}}}{K_{p \text{ target}}} \quad (2)$$

The holdup volume, which is the volume of the solution occupying the voids in the resin matrix, was taken into account in the determination of the K_p .

4.2.4 High-throughput miniature column experiments

To determine the optimal point of antibody separation from HCP impurities, design of experiments (DoE) was performed using 600 μL miniature columns packed with either POROSTM XS (Thermo Fisher Scientific) or Capto adhere (Cytiva). A surface response model with a quadratic I-optimal design and 16 runs was employed to optimize the separation conditions for miniature columns. The two factors investigated were pH and counterion concentration, while the three responses were $\log(K_p)$ HCP, $\log(K_p)$ mAb and $\log(\alpha)$. The center point for each experiment was selected based on the areas with the lowest separation factor α for CEX and the highest for MMC observed in batch-binding screenings, reflecting the application of CEX in bind/elute mode and MMC in flow-through mode. To simulate lab-scale column chromatography conditions, miniature columns were equilibrated with 5 CV of equilibration buffer at a flow rate of 200 $\mu\text{L}/\text{min}$. They were then loaded with 5 CV of buffer-exchanged sample at 120 $\mu\text{L}/\text{min}$, washed with 1 CV of equilibration buffer at 200 $\mu\text{L}/\text{min}$, and eluted with 3 CV of strip buffer at 300 $\mu\text{L}/\text{min}$. Prior to the experiment, mAb or HCP samples were buffer exchanged into the respective equilibration buffer using a 53 mL HiPrep 26/10 desalting column and further diluted to achieve a load challenge of 12.5 g/L resin for mAbs and < 4 g/L resin for HCPs. Concentrations of all fractions and loads were determined by measuring absorbance at 280 nm in triplicates. Statistical analysis was performed using Design Expert (version 13.05.0). Analysis of variance (ANOVA) was initially performed for model construction by including all significant parameters having a $p \leq 0.05$. Model quality was assessed using the model F-value, lack of fit value, and adjusted R^2 statistics.

4.2.5 Lab-scale verification experiments

To verify the transferability of the optimal separation conditions identified in the miniature column DoE experiments for mAb and HCP, validation experiments were performed on the Äkta Avant 25 system using both HiTrap Capto adhere (Cytiva) and POROSTM GoPureTM XS 1 mL columns (Thermo Fisher Scientific). Samples were buffer exchanged and diluted to achieve a loading of 37.5 g/L resin for mAbs and 2.5 g/L resin for HCPs. Peak fractions were pooled, and the specific concentrations of HCP and mAbs were quantified individually. Two different biolayer interferometry (BLI) assays were used for these measurements: an in-house developed assay targeting recombinant hydrolases for HCP quantification, and an antibody quantification assay using Protein A sensors.

4.2.6 Biolayer interferometry for quantification of IgGs and specific HCPs

IgGs and HCPs were specifically quantified using BLI on an Octet Red384 instrument (ForteBio). Protein A sensors were used for IgG quantification according to the manufacturer’s instructions. The procedure included five steps: 1. Pre-hydration (10 min), 2. Baseline (60 s), 3. Sample loading (30 s), 4. Regeneration (30 s) with 10 mM glycine buffer (pH 1.5), and 5. Neutralization (30 s). Specific HCPs were quantified using an in-house developed assay against recombinant proteins. This assay measures in a linear quantification range of 10-300 ng/mL. Anti-His biosensors were pre-hydrated, and the BLI method involved eight steps, including loading, washing, and antibody binding. Quadruplicate measurements were performed for lab-scale validation experiments. Data acquisition and analysis were performed using the Octet Data Acquisition Software 9.0 and Data Analysis Software 11.1, respectively.

4.3 Results

4.3.1 Binding behavior of critical host cell proteins in the polishing steps

High-throughput binding screenings were performed to investigate the binding behavior of the polysorbate degrading HCPs CES1F, CES2C, LPLA2 and PAF-AH. These recombinantly expressed HCPs were first purified using a three-step process consisting of two capture steps (Ni-NTA and StrepTactin), followed by size exclusion chromatography. The primary output of the binding screenings, the partition coefficient (Kp), served as a key indicator of HCP binding or flow-through under specific pH and counter ion concentration conditions. Each screening generated 48 unique data points, providing a comprehensive insight into specific HCP behavior under various conditions.

The binding behavior of CES2C, LPLA2, PAF-AH, and CES1F was investigated on the CEX resin Poros XS, using a combination of eight sodium counter ion concentrations (38-500 mM) and six pH levels (4-8). Figure 4.1 shows contour plots to illustrate the logarithmic Kp for each polysorbate degrading hydrolase, represented across a range of pH levels and Na⁺-counterion concentrations (mM). All HCPs examined displayed a higher sensitivity to pH than Na⁺-counter ion concentration, as indicated by the vertically oriented black contour lines defining the HCP partitioning regions on the resin. The impact of Na⁺-counter ions on resin binding was more significant for PAF-AH (Figure 4.1C) than for CES2C (Figure 4.1A), as indicated by the more horizontal shifts of the strong binding contour line ($\log(Kp) = 1$) for LPLA2 and PAF-AH in the contour plots. The contour plots also show that HCPs exhibiting higher pI values, such as PAF-AH (pI = 8.6), demonstrated a shift in the strong binding region towards higher pH ranges compared to CES2C (pI = 5.8). CES2C is the only polysorbate degrading hydrolase with a parallel transition of partitioning regions, where a shift from strong binding to flow-through occurs within a pH range of 0.5. LPLA2 showed two distinct flow-through regions at pH > 7 (Figure 4.1B), while CES1F (Figure 4.1D) shows a small flow-through area at pH higher than 7.75 and Na⁺-

counter ion concentrations below 100 mM. Notably, PAF-AH has no flow-through region in the tested ranges (Figure 4.1C).

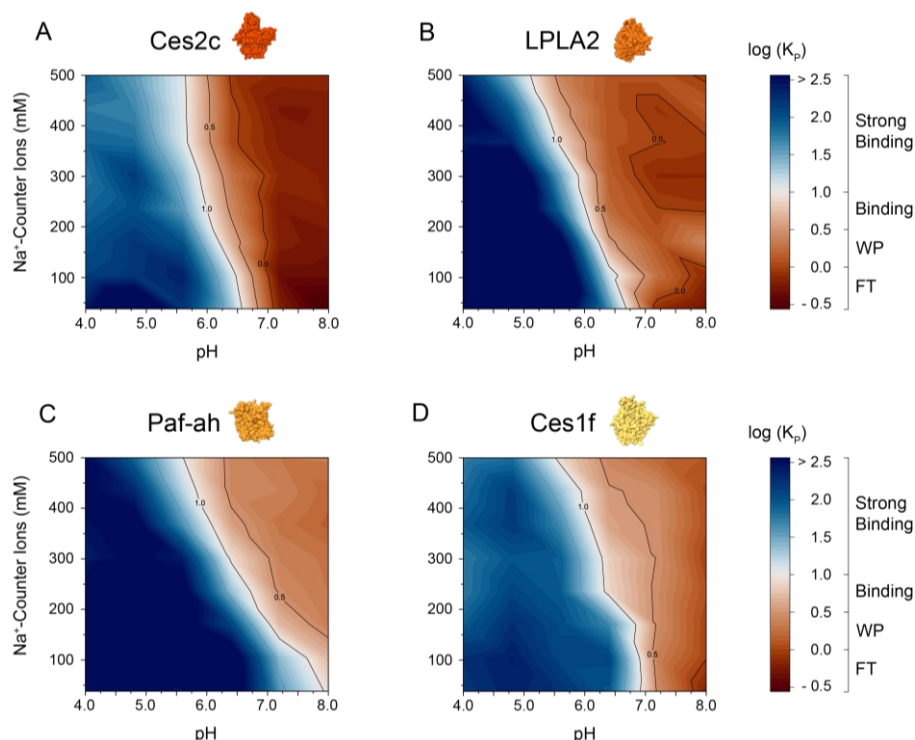


Figure 4.1: High-throughput polysorbate hydrolase binding screening on Poros XS resin. The contour plots illustrate the binding behavior of polysorbate hydrolases during bind-and-elute cation exchange chromatography, with $\log(K_p)$ values represented across varying pH levels and concentrations of Na^+ -counter ions (mM) for (A) CES2C, (B) LPLA2, (C) PAF-AH, and (D) CES1F. Binding affinity to the resin is represented by $\log(K_p)$ values, categorized as: < 0 (flow-through, FT), $0 \leq 0.5$ (weak partitioning, WP), $0.5 \leq 1.0$ (binding), and > 1.0 (strong binding).

The binding properties of CES2C, LPLA2, PAF-AH and CES1F on the MMC resin Capto adhere were examined using eight distinct chloride counter ion concentrations (60-1000 mM) and six pH levels (4-10). The logarithmic K_p for each HCP, as a function of pH and Cl^- -counter ion concentration, showed unique binding patterns on mixed mode resin as shown in contour plots (Figure 4.2). CES2C binds strongly to the resin over the entire range tested, except for a narrow pH range of 4-5.2 and below 500 mM Cl^- -counter ions (Figure 2A). With increasing salt concentrations, hydrophobic interactions may intensify, leading to stronger binding in this area. Conversely, LPLA2 and CES1F exhibit greater sensitivity to pH than to Cl^- -counter ion levels with respect to binding transitions (Figure 4.2B and D). PAF-AH shows salt sensitivity evidenced by its tilted oval-shaped strong binding contour line (Figure 4.2C). Here, electrostatic repulsion seemingly outweighs hydrophobic interaction, resulting in weakened resin-protein interactions at salt concentrations above 500 mM.

These results indicate that the binding behavior of polysorbate degrading hydrolases to Capto adhere resin varies and lacks common trends as observed with CEX resin POROS™ XS.

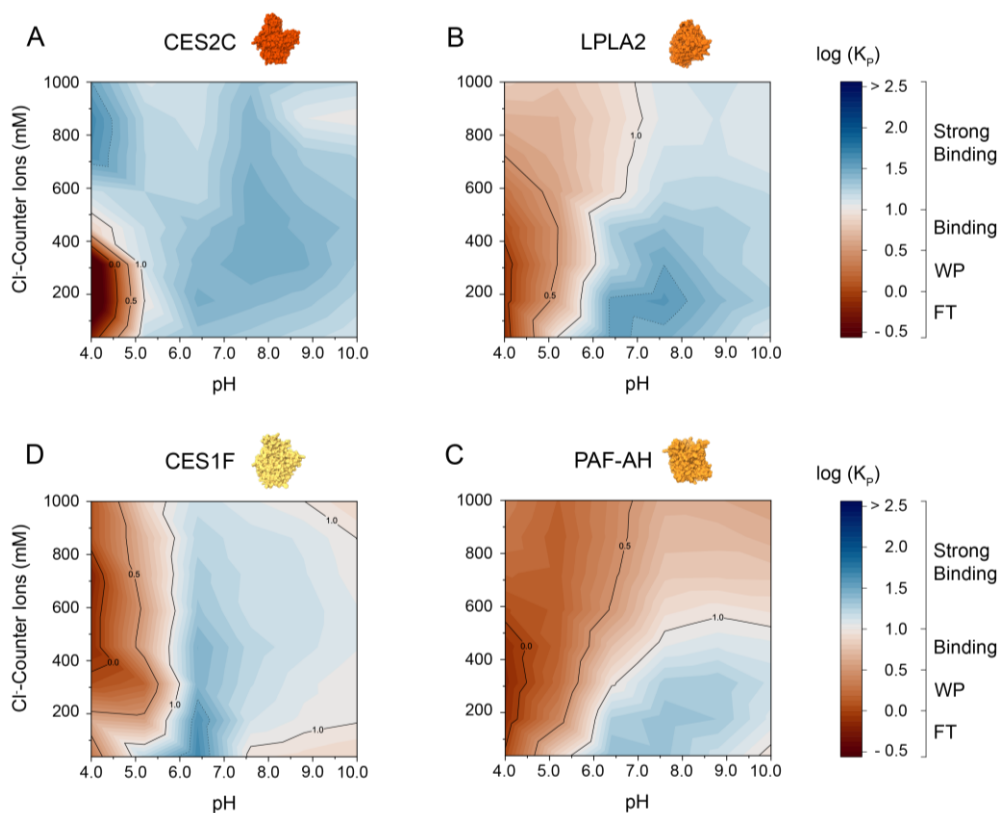


Figure 4.2: High-throughput polysorbate hydrolase binding screening on Capto adhere resin. Contour plots illustrate the binding behavior of polysorbate hydrolases during flow-through mixed-mode chromatography, with $\log(K_p)$ values represented across varying pH levels and concentrations of Cl-counter ions (mM) for (A) CES2C, (B) LPLA2, (C) CES1F, and (D) PAF-AH. Binding affinity to the resin is indicated by $\log(K_p)$ values, with ranges: < 0 (flow-through, FT), $0 \leq 0.5$ (weak partitioning, WP), $0.5 \leq 1.0$ (binding), and > 1.0 (strong binding).

4.3.2 Behavior of mAbs in the polishing steps

To tackle the challenge of separating the HCP impurities from the final mAb product, it is vital to compare the binding profiles of mAbs and specific HCPs under identical screening conditions, thereby pinpointing differences in their binding behaviors. The high-throughput screenings were performed for various antibody formats under the same conditions as for the polysorbate degrading hydrolases. Both cation exchange and mixed mode screenings were performed. The cation exchange resin contour plots, illustrating the logarithmic K_p as a function of pH and Na^+ concentration, revealed a decreasing requirement of Na^+ ions for mAb binding with increasing pH (Figure 4.3). In particular, mAb-5 showed robust binding over the entire pH screening range when Na^+ counterion concentrations were maintained below 250 mM (Figure 4.3D). These results indicate that the Na^+

counterion concentration has a greater influence on mAb-resin interactions than pH during CEX chromatography.

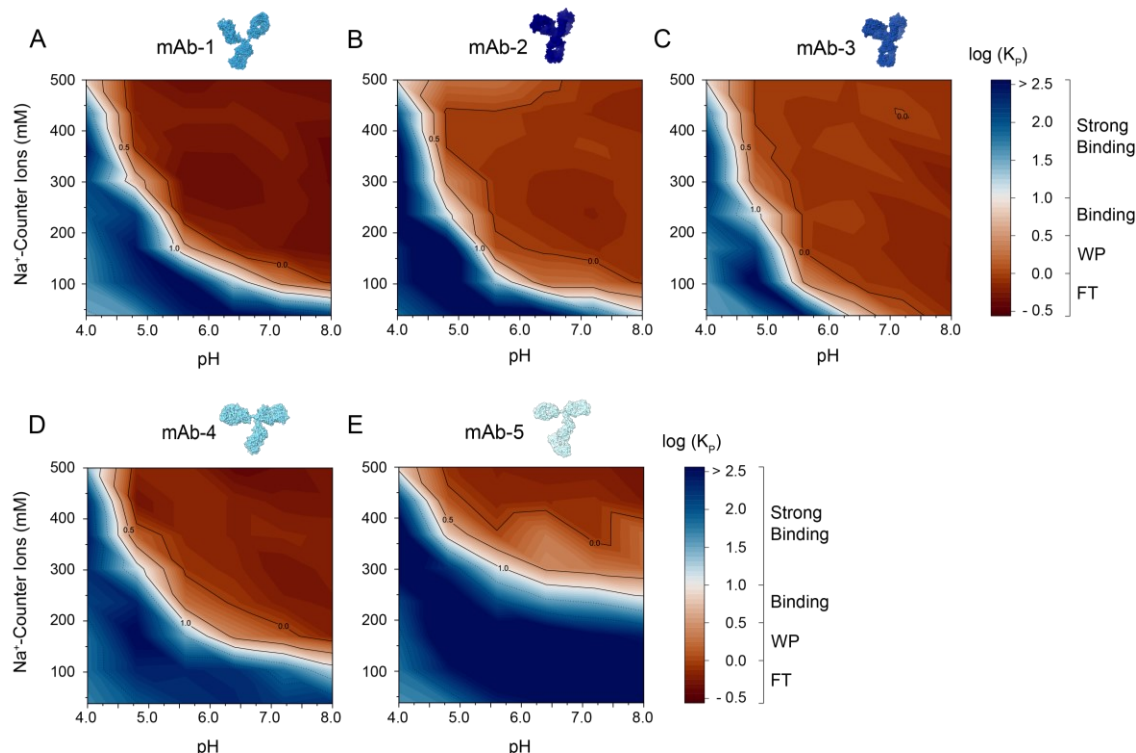


Figure 4.3: High-throughput mAb binding screening on Poros XS resin. Contour plots illustrate the binding behavior of mAbs during bind-and-elute cation exchange chromatography, with log (K_p) values represented across varying pH levels and concentrations of Na⁺-counter ions (mM) for (A) mAb-1 (IgG1), (B) mAb-2 (IgG4), (C) mAb-3 (IgG4), (D) mAb-5 (IgG-scFv), and (E) mAb-4 (bispecific-mAb). The binding affinity to the resin is represented by log (K_p) values, categorized as: < 0 (flow-through, FT), 0 ≤ 0.5 (weak partitioning, WP), 0.5 ≤ 1.0 (binding), and > 1.0 (strong binding).

Antibody binding was also examined on the mixed mode (MMC) resin (Figure 4.4). The IgG1 (mAb-1), IgG-scFv (mAb-5), and bispecific-mAb (mAb-4) displayed a partially oval shaped transition from flow-through to strong binding, indicating that the binding behavior is almost equally influenced by pH and Cl⁻-counter ion concentration, but the size of each partitioning area depended on the molecule (Figure 4.4 A, D, and E). In contrast, the binding behavior of the tested IgG4-subtypes was more sensitive to pH levels than the concentration of Cl⁻-counter ions (Figure 4.4 B and C). In particular, the two tested IgG4 mAbs showed a narrow flow-through area within the examined pH range near pH 4 and a Cl⁻-counter ion concentration below 200 mM. These results highlight that, similar to HCPs, the binding behavior of mAbs to MMC resin varies from molecule to molecule, with no global trends as observed for CEX resin.

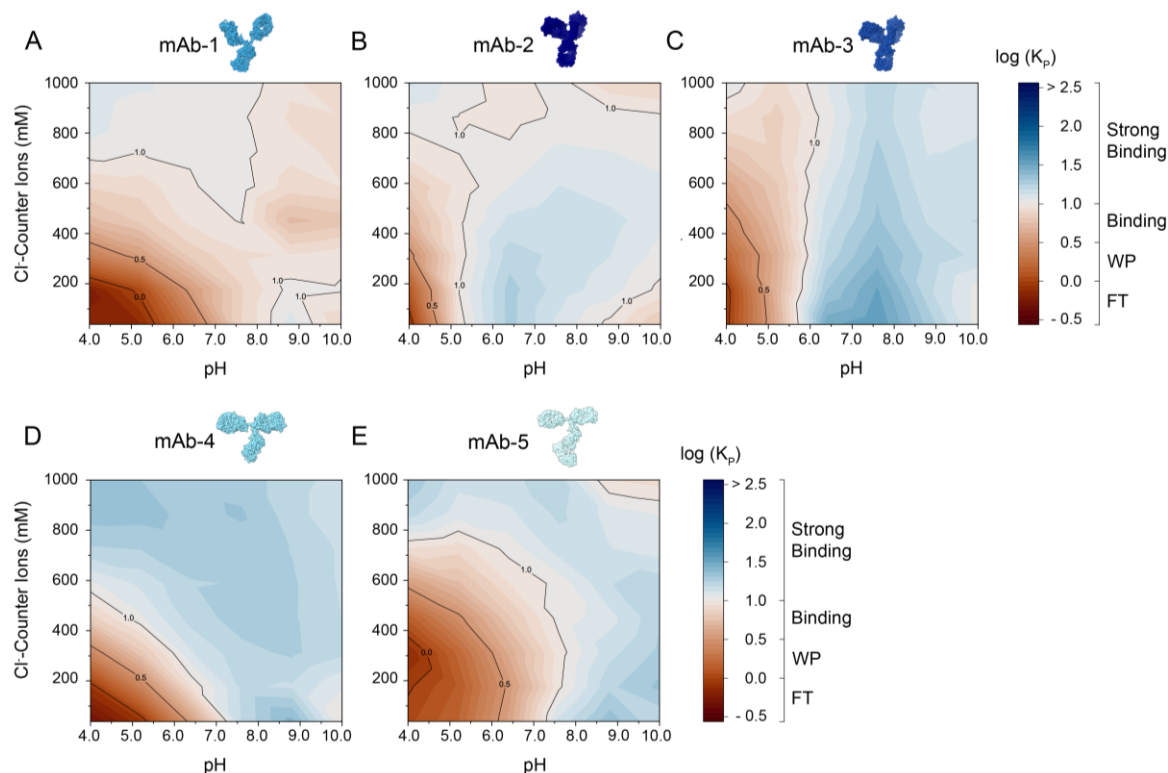


Figure 4.4: High-throughput mAb binding screening on Capto adhere resin. Contour plots illustrate the binding behavior of mAbs during flow-through mixed mode chromatography, with $\log(K_p)$ values represented across varying pH levels and concentrations of Cl-counter ions (mM) for (A) mAb-1 (IgG1), (B) mAb-2 (IgG4), (C) mAb-3 (IgG4), (D) mAb-5 (IgG-scFv), and (E) mAb-4 (bispecific-mAb). The binding affinity to the resin is represented by $\log(K_p)$ values, categorized as: < 0 (flow-through, FT), $0 \leq 0.5$ (weak partitioning, WP), $0.5 \leq 1.0$ (binding), and > 1.0 (strong binding).

4.3.3 Separation of specific host cell proteins from mAbs

To identify optimal separation conditions for specific mAbs and polysorbate degrading hydrolases, the separation factor α was calculated for each combination of pH and counterion concentration. The binding behavior of four HCPs and five mAbs was investigated on two chromatography resins (POROSTM XS and Capto adhere). This comprehensive analysis resulted in a total of 40 separation factor α contour plots, all of which are presented in the Supplementary Figures (Figure B1 and Figure B2). In this section, we present a selection of these plots that best illustrate our key findings. The POROSTM XS CEX resin operates in bind-elute mode, requiring mAb binding to the resin and HCP flow-through. Separation conditions are indicated by $\log(\alpha)$ values, with lower values indicating better separation. In this mode, mAb binding to the resin ($\log(K_p) > 0.5$) and HCP flow-through ($\log(K_p) < 0$) are required. $\log(\alpha)$ values below zero indicate improved separation, while values above zero indicate no separation between mAb and HCP. The selected $\log(\alpha)$ contour plots for mAb-5 and four HCPs (CES2C, LPLA2, PAF-AH, and CES1F) are shown in Figure 4.5. A broad separation range for CES2C, LPLA2, and CES1F is observed at pH values above 6 and Na⁺-counter ion concentrations below 250 mM, with a

$\log(\alpha)$ range of -1.3 to -2.5 (Figure 4.5 A, B, and D). For PAF-AH, the $\log(\alpha)$ range of -1.3 to -1.9 is limited to pH values above 7 and Na^+ -counter ion concentrations below 225 mM (Figure 4.5 C). This highlights the sensitivity of the interaction forces between the mAb and the resin to Na^+ counter ions, and the influence of pH on the complex MMC interactions of the HCP, thereby creating a separation window. However, this separation window is not present for mAb-3 due to the lack of strong binding at pH 6.26 (Figure 4.3). These results suggest that suitable separation conditions cannot be determined here for all HCPs tested, especially CES2C and LPLA2 (Figure 4.6).

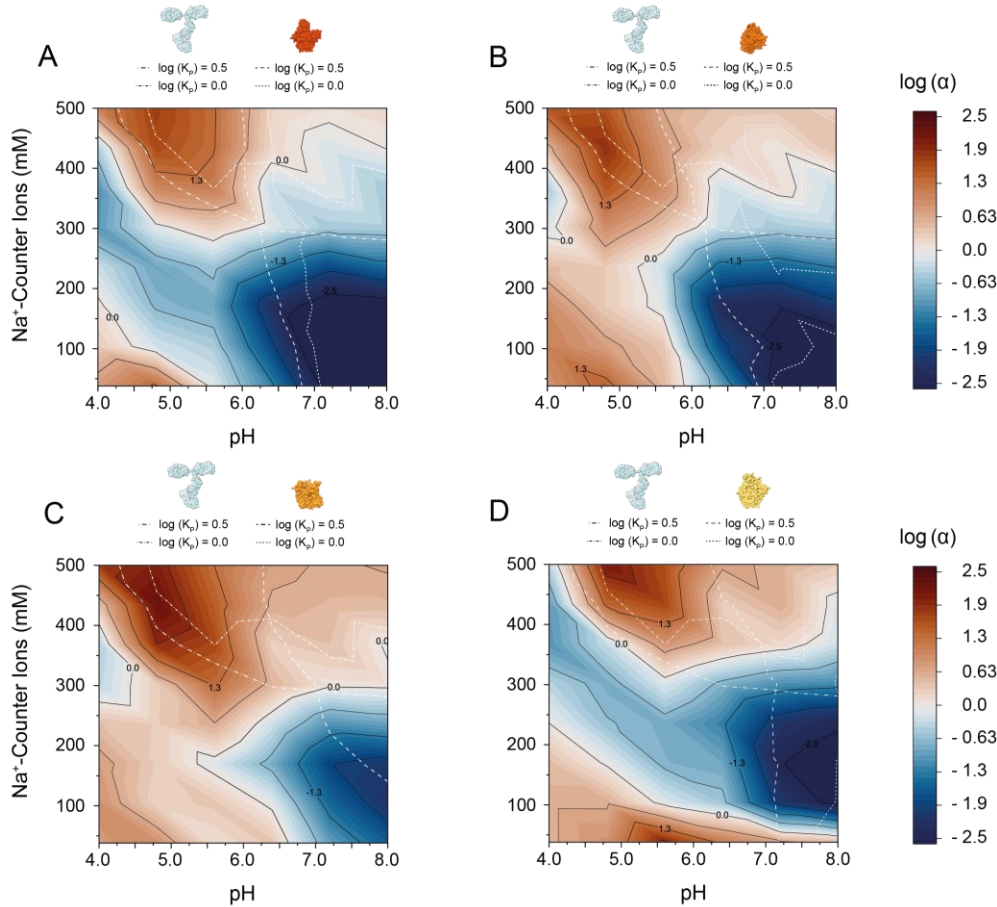


Figure 4.5: Identification of cation exchange separation conditions between mAb-5 and four polysorbate hydrolases. Contour plots show the logarithmic separation factor α ($\log(\alpha)$) as a function of pH and Na^+ -counter ions (mM) for the target protein mAb-5 (IgG-scFv) and the HCP impurities (A) CES2C, (B) LPLA2, (C) PAF-AH and (D) CES1F. In case of bind-and-elute cation exchange chromatography mAb and HCP separation is indicated by decreasing $\log(\alpha)$ values < 0 (white to dark blue). The white lines indicate the weak partitioning region of each protein with $\log(K_p)$ values of 0 and 0.5. $\log(K_p) = 0$: transition line between flow-through and weak-partitioning (mAb-5: short dashed-dotted; HCP: short dashed), $\log(K_p) = 0.5$: transition from weak partitioning to binding (mAb-5: dashed-dotted; HCP: dashed).

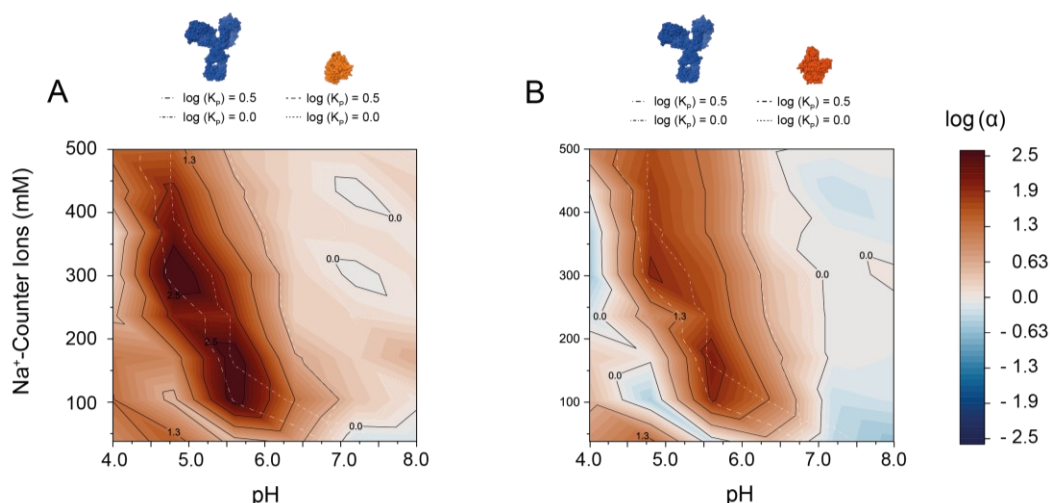


Figure 4.6: Identification of cation exchange separation conditions between mAb-3 and two polysorbate hydrolases. Contour plots show the logarithmic separation factor α ($\log(\alpha)$) as a function of pH and Na^+ -counter ions (mM) for the mAb-3 and the polysorbate hydrolases (A) LPLA2 and (B) CES2C.

The Capto adhere MMC (mixed mode) resin operates in flow-through mode and requires mAb flow-through ($\log(K_p) < 0$) and HCP binding ($\log(K_p) > 0.5$). MAb and HCP separation is indicated by $\log(\alpha)$ values above zero, with higher values indicating better separation. $\log(\alpha)$ values below zero indicate no separation. Figure 4.7 shows $\log(\alpha)$ contour plots for mAb-1 (IgG1) and the four HCPs as a representative example. PAF-AH shows weak separation ($\log(\alpha) < 0.75$) (Figure 7C). The other three polysorbate degrading hydrolases showed a narrow separation range ($\log(\alpha) > 1.1$) around pH 6 and 100 mM Cl^- concentration (Figure 4.7 A-C). The Capto adhere resin exhibited a narrower separation window ($\log(\alpha) > 1$) compared to the CEX resin, which corresponds to the proximate partitioning areas for HCPs and mAbs. These observations highlight that, within MMC, there is a broad spectrum where $\log(\alpha) > 0$, but achieving optimal separation requires $\log(\alpha)$ values greater than 1.0. This is limited by the overlapping partitioning regions of HCP and mAb. An example of this overlap can be observed between mAb-2 and CES2C (Figure B2).

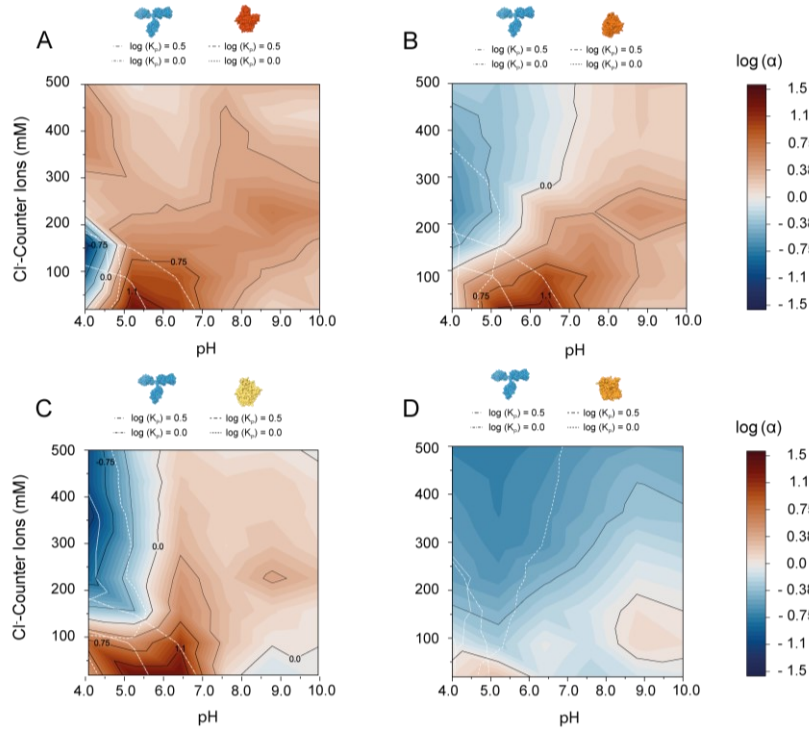


Figure 4.7: Identification of mixed mode separation conditions between mAb-1 and polysorbate hydrolases. Contour plots display the logarithmic separation factor α ($\log(\alpha)$) as a function of pH and Cl-counter ions (mM) for mAb-1 and the polysorbate hydrolases (A) CES2C, (B) LPLA2, (C) PAF-AH and (D) CES1F. In case of mixed-mode chromatography mAb and HCP separation is indicated by increasing $\log(\alpha)$ values > 0 (white to dark red). The white lines indicate the weak partitioning region of each protein with $\log(K_p)$ values of 0 and 0.5. $\log(K_p) = 0$: transition line between flow-through and weak-partitioning (mAb-1: dashed-dotted; polysorbate hydrolase: dashed), $\log(K_p) = 0.5$: transition from weak partitioning to binding (mAb-1: short dashed-dotted; HCP: short dashed).

4.3.4 Separation of specific host cell proteins from mAbs on miniature columns

In order to transfer the identified separation regions to a packed bed column format and to improve the separation conditions, a surface response model with a quadratic I-optimal design was applied to 600 μL miniature columns. This was investigated for two distinct mAb-HCP combinations: mAb-5 (IgG-scFv) with CES2C on CEX resin, and mAb-1 (IgG1) with LPLA2 on MMC resin.

In the first scenario, involving mAb-5 (IgG-scFv) and CES2C, a 16-run design was utilized, with a screening range of 50 to 250 mM Na^+ -counter ions and pH values between 5.75 and 7.75. The most favorable separation condition was identified at pH 7.28 and 151.4 mM Na^+ -counter ions, where the antibody showed strong binding to the resin while the polysorbate degrading hydrolase was found in the flow-through. This optimal separation condition, as shown in Figure 4.8, met the criteria for the lowest $\log(K_p)$ CES2C, lowest $\log(\alpha)$, and a high $\log(K_p)$ for mAb-5, indicated by the red circle.

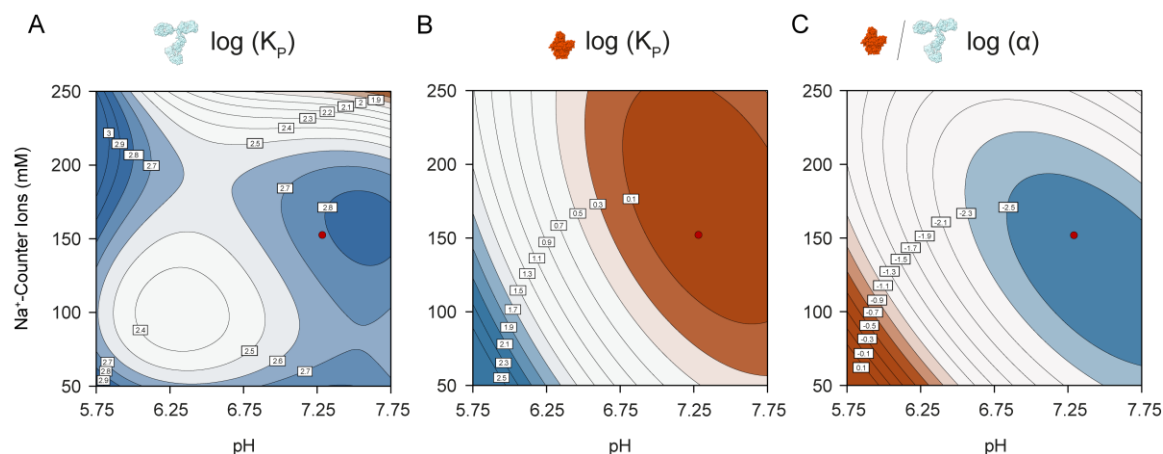


Figure 4.8: Determination of the optimal separation conditions for mAb-5 and CES2C on Poros XS miniature columns. Contour plots illustrate the influence of pH and Na⁺-counter ions (mM) on the $\log(K_p)$ of the target protein mAb-5 (A), the $\log(K_p)$ of impurity CES2C (B), and the logarithmic separation factor $\log(\alpha)$ (C), based on a quadratic I-optimal design. The red dot represents the optimal separation condition for mAb-5 and CES2C.

For the second scenario, focusing on mAb-1 (IgG1) and LPLA2, the same DoE model was implemented, using a screening range of 75 to 275 mM Cl⁻-counter ions and pH values ranging from 4.75 to 6.75, derived from previous high-throughput binding screening. The MMC resin Capto adhere operates in flow-through mode, requiring mAb flow-through ($K_p < 0$) and strong HCP binding to the resin ($K_p > 1$) for packed bed columns. LPLA2 and mAb-1 displayed higher sensitivity to pH than to Cl⁻-counter ions within this screening range, as evidenced by the vertically oriented contour lines (Figure 4.9 A and B). The design identified an optimal separation region where $\log(\alpha)$ is greater than 2.0, specifically for pH values ranging from 5.9 to 6.2 and Cl⁻-counter ions less than 100 mM (Figure 4.9 C). The optimal separation condition was determined to be at pH 5.87 and 107 mM Cl⁻-counter ions, satisfying the requirements of a $\log(K_p)$ below 0 for mAb-1, a maximized $\log(K_p)$ for LPLA2, and a maximized $\log(\alpha)$.

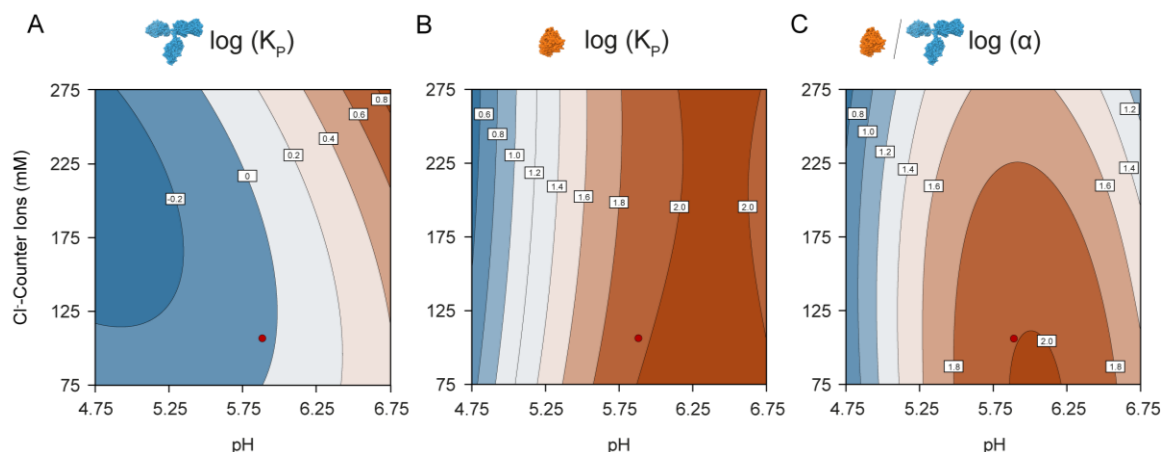


Figure 4.9: Determination of the optimal separation conditions for mAb-1 and LPLA2 on Capto adhere miniature columns. Contour plots illustrate the influence of pH and Cl⁻ counter ions (mM) on the $\log(K_p)$ of the target protein mAb-1 (A), the $\log(K_p)$ of the impurity LPLA2 (B), and the logarithmic separation factor $\log(\alpha)$ (C), using a quadratic I-optimal design. The red dot represents the optimal separation condition for mAb-1 and LPLA2.

4.3.5 Verification of host cell protein and mAb separation conditions on lab-scale columns

The final step of our study was to verify mAb and polysorbate degrading hydrolase separation conditions using packed bed columns on a chromatography system. To assess the potential impact of the presence of mAb on the binding and elution behavior of polysorbate degrading hydrolases due to non-specific binding, both components were evaluated simultaneously and individually for comparison. This verification was performed specifically for mAb-5 and CES2C using Poros XS cation exchange and for mAb-1 and LPLA2 using Capto adhere mixed mode.

The optimal separation conditions for mAb-5 and CES2C on a POROSTM XS column were verified based on results from the miniature experiments at pH 7.28 and 151.4 mM Na⁺. Under these separation conditions, 98.7 % of CES2C was separated from mAb-5 (Figure 4.10). The presence of mAb-5 slightly increased the survival rate of CES2C. However, no significant differences were observed between the combination of mAb-5 and CES2C in the control runs. The majority of mAb-5 was eluted at satisfactory levels in the salt gradient for mAb-5 and CES2C in combination (96.5 %) and the mAb alone (98.5 %).

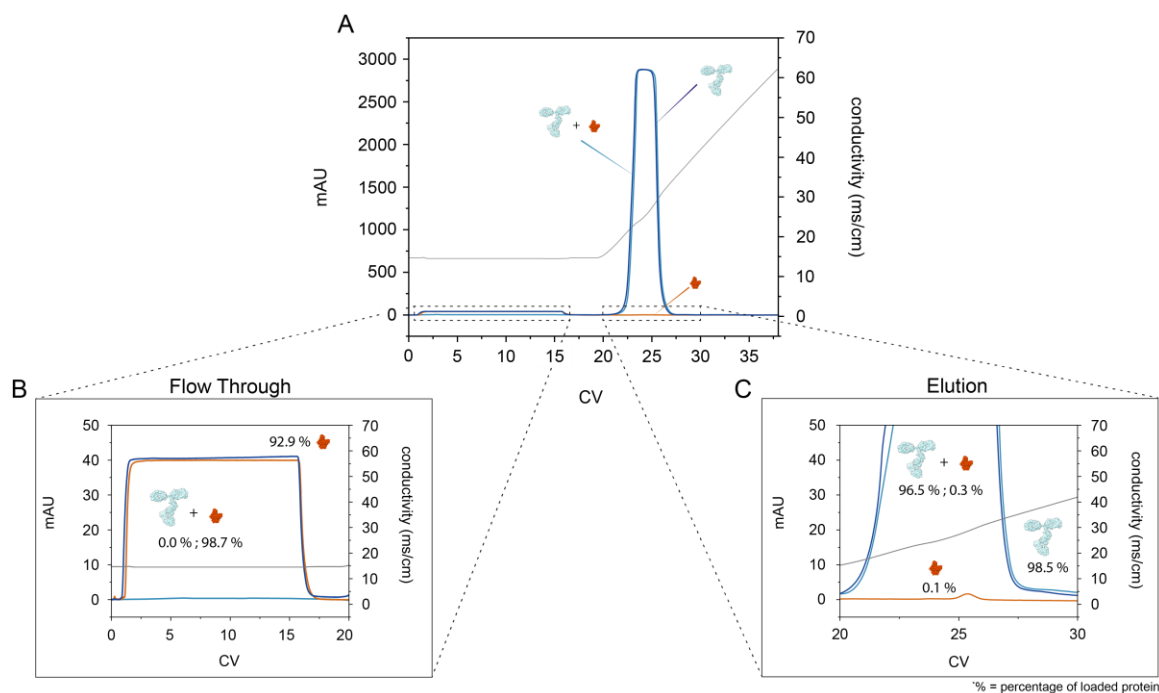


Figure 4.10: Verification of separation conditions for mAb-5 and CES2C using a 1 mL POROS XS column. Chromatogram (A) shows cation exchange chromatography runs in bind-and-elute mode for mAb-5 and CES2C combined (cyan), mAb-5 alone (blue), and CES2C alone (orange). The flow-through (B) and elution (C) fractions show the percentage of loaded protein. Conductivity (mS/cm) is shown in gray. Flow-through: 0-18 column volumes (CV), wash: 18-20 CV, gradient elution: 20-38 CV.

The verification for mAb-1 and LPLA2 was performed on a MMC column (Capto adhere) at pH 5.87 and 107 mM Cl⁻ counter ions. The generated chromatograms included the combined mAb-1 and LPLA2, as well as each molecule individually (Figure 4.11). Remarkably, 99.3 % of LPLA2 was separated from the antibody, with no significant changes observed in the flow-through fractions for the HCP LPLA2, whether in combination with the antibody or alone. An antibody recovery rate of 99.3 % was achieved. Interestingly, the majority of LPLA2, which exhibited strong binding to the resin, was not eluted during the salt gradient process but was instead found during the column cleaning steps. These results suggest that the separation conditions identified in the miniature column experiments can be successfully transferred to the packed bed columns. Furthermore, the data suggest that the presence of the mAb-1 has no significant impact on LPLA2 levels in the flow-through fraction.

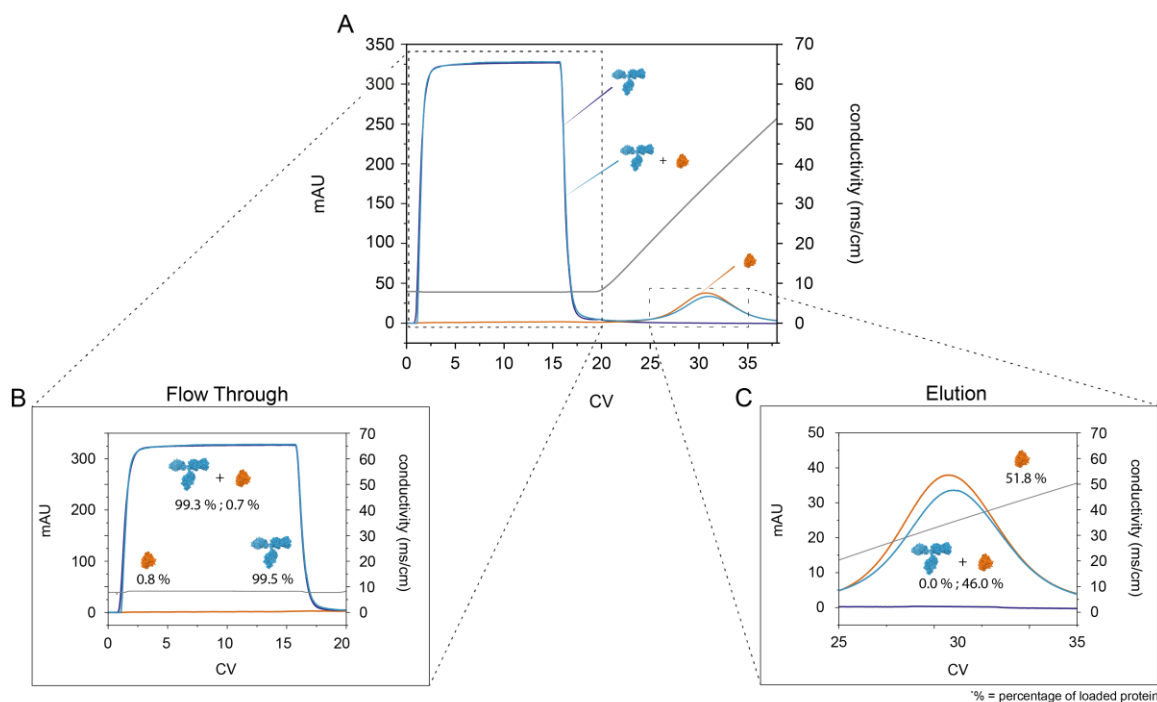


Figure 4.11: Validation of separation conditions for mAb-1 and LPLA2 using a 1 mL Capto adhere column. Chromatograms (A) show flow-through mixed mode chromatography runs for mAb-1 and LPLA2 combined (cyan), mAb-1 alone (blue), and LPLA2 alone (orange). Flow-through (B) and elution (C) fractions show the percentage of loaded protein. Conductivity (mS/cm) is shown in gray. Flow-through: 0-18 CV column volumes (CV), wash: 18-20 CV, gradient elution: 20-38 CV.

4.4 Discussion

The objective of this study was to address two major gaps in the current understanding of polysorbate degrading HCP behavior during downstream processing, focusing specifically on cation exchange and mixed-mode chromatography as polishing steps. The first gap concerns the limited knowledge of the binding behavior of the specific HCPs CES2C, LPLA2, PAF-AH and CES1F, to the different resin types. The second gap concerns the identification of separation conditions for effective removal of critical HCPs from the antibody. Bridging these gaps is critical as polysorbate degrading HCPs continue to be identified in antibody formulations, highlighting the need for improved understanding and optimization of the purification process [83,106]. With respect to the binding behavior of polysorbate degrading HCPs and mAbs during the polishing steps of a downstream purification process, our results illustrated different patterns under the investigated conditions. For CEX, we observed that pH has a more significant effect on the interactions between polysorbate degrading hydrolases and the resin compared to the concentration of counter ions. Interestingly, these results are consistent with McDonald et al., who found strong binding over the pH range of 5 to 6.5 and Na^+ -counter ion concentrations of 50 to 225 mM for general HCP content [139]. This suggest that our findings are consistent with the overall HCP behavior. Binding screenings of mAbs using CEX revealed a pattern that as pH

increases, a reduced number of Na^+ -counter ions are required for the mAb to shift from strong binding to moderate binding (Figure 4.3). This observation is consistent with several studies that have highlighted the more significant effect of Na^+ -counter ion concentration on mAb-resin interactions relative to pH [97,98,156]. The study by Welsh and co-workers further supports the approach of this research, showing that different loading challenges (5-120 g/L Resin) had minimal effect on the overall observed binding behavior of mAbs to Poros XS [219]. Notably, this resin specificity is not limited to Poros XS, as similar behavior was observed by McDonald et al. for the CEX resins Poros50HS and SPSFF [139]. These findings suggest that the results of this study may also be applicable to other CEX resins. Another notable observation supported by literature, is that the pI of a mAb cannot reliably predict the exact rank order in which antibodies shift from binding to non-binding conditions [124]. For example, although the two IgG4 molecules have pI values within a similar range, mAb-2 has a broader range in which it strongly binds the resin than mAb-3 (Figure 4.3). However, a general trend was observed that antibodies with higher pIs exhibited a broader range of strong binding, facilitating their separation from similarly charged polysorbate degrading hydrolases. On the other hand, antibodies with lower pIs may present challenges during purification from similarly charged polysorbate degrading hydrolases. This was evident in the case of mAb-3, where no separation window was identified in this process step. For the removal of polysorbate degrading HCPs by CEX, higher pIs are generally more favorable.

When mixed-mode chromatography resins were used, the binding behavior of polysorbate degrading hydrolases showed significant variation among individual molecules, with no discernible general trends.

These observations highlight the importance of understanding the unique binding characteristics of individual HCPs to optimize downstream processing steps, especially when targeting the separation of a specific polysorbate degrading hydrolase. The results of this study suggested that polysorbate degrading hydrolases may persist through polishing steps without specific tailoring of column conditions due to similar binding behavior. In both CEX and MMC for mAbs and the polysorbate degrading hydrolases analyzed, a consistent trend shows that optimal separation occurs at low counter-ion concentrations (60 to 150 mM), which is consistent with the findings by Kelley and colleagues for overall HCP removal [97]. Regarding pH, the ideal range for CEX was determined to be typically above 6.5, while for MMC, it is between 5.5 and 6.5 (Figure 4.1 and Figure 4.2).

This study also provides insight into how a three-step strategy can identify downstream separation opportunities demonstrated for two different mAb-HCP combinations. The identified separation conditions were successfully validated on packed bed columns, achieving satisfactory reduction of CES2C in CEX and LPLA2 in MMC. In this context, it is important to note that the quantification of the polysorbate hydrolases was achieved using specific tags. This approach was necessitated by the lack of commercially available antibodies specific to native CHO proteins. Furthermore, accurately measuring individual HCPs in the presence of a mAb at extremely low levels (below 10 ppm) via mass spectrometry presents significant challenges. Given the relatively small size of the His- and

Strep-tag compared to the HCPs, their influence on partitioning behavior is considered negligible. These tags, composed of short amino acid sequences, are unlikely to substantially alter the hydrophobicity or charge distribution of the proteins, thereby not affecting their binding characteristics in cation exchange and mixed mode chromatography. Although the presence of the tags could induce slight shifts in the weak partitioning region, the determined separation conditions – with $\log(K_p)$ values less than 0 for CEX and greater than 1 for MMC – are sufficiently distinct from this range. Thus, even if minor shifts in the weak partitioning region occur, effective separation is still achieved.

Interestingly, the data suggest that co-elution, rather than hitchhiking, seems to be the main reason for the co-purification of certain polysorbate degrading hydrolases (and maybe also other HCPs) in the downstream process. Hitchhiking involves non-specific binding of HCPs to mAbs or other target molecules during purification, whereas co-elution occurs when mAbs and HCPs have similar properties, causing them to elute together. The study by Hecht et al. found that LPLA2 has low affinity for IgG1 and IgG4 antibodies, which could contribute to polysorbate hydrolase survival through the downstream processing. [75] Our results for LPLA2 and mAb-1 (IgG1) on MMC suggest that low affinity does not cause additional LPLA2 to elute (Figure 4.11). A minimal effect of hitchhiking on the screened polysorbate degrading hydrolases is supported by CEX chromatography column runs with CES2C and mAb-5. Another study by Zhao et al. investigated the separation of different HCPs through size exclusion chromatography. The polysorbate degrading hydrolase CES1F could be separated from the mAb under native conditions, indicating no effective binding between the mAb and this hydrolase [241].

Determining the optimal separation factor for all mAbs, especially for mAb-3 (IgG4) in this study, can be challenging. The binding behavior of mAb-3 in MMC and CEX is largely consistent with that of the four polysorbate degrading hydrolases studied (Figure B1 and Figure B2). Since separation depends on the different binding behavior of the HCPs and the target mAb to the resin, similar binding patterns result in co-elution. The efficiency of separation of specific HCPs from mAbs during the MMC and CEX steps is strongly influenced by the molecular properties of the mAbs. The results of this study indicate that complete removal of polysorbate degrading hydrolases might not be possible for all mAb-HCP combinations. Hence, alternative approaches are needed to achieve further removal of polysorbate degrading hydrolases. This could include the optimization of the capture step. The observed co-elution during the polishing steps does not explain the persistence of HCPs during the capture steps. Some studies have suggested that HCPs may also associate with aggregates [83], or survive the capture step due to mAb cluster formation [125,147]. However, these possibilities are beyond the scope of this publication. Optimizing the capture step involves optimization in binding and wash conditions, reducing the burden on subsequent polishing steps. However, it must be ensured that the antibody remains structurally intact, and the yield is maintained under these conditions. Another potential approach to remove particularly critical polysorbate degrading hydrolases from certain mAbs could be to modify the antibody backbone to alter the physicochemical properties of the mAb. This modification might potentially prevent co-elution, thereby improving the separation and purification process. However, this approach requires careful consideration

of the potential impact on the antibody’s efficacy and stability. Structural modifications might affect the antibody’s binding affinity, half-life, and overall therapeutic performance, necessitating extensive validation. In addition, the use of cell line engineering to knock out polysorbate degrading hydrolases is a promising strategy, but several challenges must be addressed. Knocking out specific hydrolases could lead to compensatory upregulation of other hydrolases, potentially affecting cell viability, growth rates or protein production. Some hydrolases may play critical roles in cellular functions, making their knockout problematic. Extensive validation is required to ensure the modified cells perform equivalent to their non modified counterparts. To date, this approach has so far only been successfully demonstrated for the removal of lipoprotein lipase (LPL) in CHO cells [32,47], indicating the need for further research to extend this approach to other hydrolases involved in polysorbate degradation.

4.5 Conclusion

Our study is the first in the field to take a detailed look at hydrolase-antibody co-elution, focusing specifically on cation exchange (CEX) and mixed-mode chromatography (MMC). Our high-throughput binding screenings revealed unique binding behaviors of various HCPs and mAbs, highlighting the importance of understanding these characteristics to optimize downstream processing. Lower counter ion concentrations were found to improve HCP clearance in both CEX and MMC. We employed a three-step strategy, consisting of high-throughput binding screenings, miniature column experiments, and packed bed column experiments, to evaluate the binding behavior of HCPs and mAbs under different conditions and identify differences in resin interaction. This approach allowed us to identify optimal separation conditions for both CEX and MMC that resulted in HCP reduction while maintaining high mAb recovery rates. However, the separation of some HCPs from certain mAbs remains challenging, indicating the need for alternative approaches. It is important to note that considering other resins and chemistries may further optimize the purification process. The variability in binding behavior, especially observed in the mixed mode resin, highlights the potential for further enhancements in the purification process through careful resin selection. Furthermore, to gain a more comprehensive understanding of the overall purification process, it is necessary to extend research efforts to other steps in the bioprocess. This will provide a more holistic view of the process and may reveal additional opportunities for optimization.

Acknowledgements

The authors would like to thank Kristina Wösle and Katharina Beingröbl for automation support and Angelika Fröschle for assistance in design of experiments planning.

5 Quantitative Analysis Reveals Hitchhiking Drives Polysorbate Hydrolase Persistence Via Host Cell Protein–Antibody Interactions

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Biotechnology and Bioengineering (2026), doi: 10.1002/bit.70170

Abstract

Polysorbate-degrading host cell proteins (HCPs) represent a critical challenge in the manufacturing of monoclonal antibody therapeutics due to their potential to persist during downstream processing. While their enzymatic activity has been characterized, the role of direct HCP-mAb interactions, particularly those involving polysorbate degrading HCPs, remains poorly understood. In this study, we systematically investigated the binding behavior of four representative polysorbate-degrading HCPs (CES1F, LPLA2, PAF-AH, PPT1) to a panel of mAbs using biolayer interferometry (BLI). All tested HCPs showed specific, transient interactions characterized by fast-on/fast-off kinetics, with apparent equilibrium dissociation constants (K_D) in the low nanomolar range (40 – 90 nM for strong binders) and rapid dissociation kinetics ($k_d > 0.01 \text{ s}^{-1}$). This indicates a binding mode characterized by relatively high affinity but limited kinetic stability.

Due to incomplete saturation and partially not meeting the quality criteria for kinetic fitting, we complemented model-based analysis with equilibrium-derived descriptors. The initial slope of the binding isotherm correlated well with kinetic parameters and enabled robust ranking of interaction strength.

To assess hitchhiking relevance during downstream processing, we performed a Protein A chromatography experiment using PLBL2 as a model HCP and two mAbs with different interaction profiles. PLBL2 levels in Protein A elution pools correlated well with interaction propensity confirming that transient interactions can contribute to HCP co-elution.

Our results provide the first systematic and quantitative comparison of polysorbate hydrolase-antibody interactions. They also demonstrate that direct mAb-HCP interaction is a relevant mechanism contributing to HCP persistence during downstream processing.

Keywords: *Host cell proteins (HCPs), polysorbate degradation, monoclonal antibodies, hitchhiking, downstream processing, biolayer interferometry*

5.1 Introduction

Polysorbates such as PS20 and PS80 are commonly used in monoclonal antibody (mAb) formulations to prevent aggregation induced by interfacial stress and to ensure long-term stability of biopharmaceutical products [99,102]. However, polysorbates are susceptible to degradation via oxidative and hydrolytic pathways, resulting in the formation of free fatty acids and visible or subvisible particles [48,92]. Enzymatic hydrolysis mediated by host cell proteins (HCPs) is among these mechanisms and has emerged as a critical risk factor for product quality of biopharmaceutical formulations [54]. Even at trace levels, polysorbate-degrading hydrolases can remain catalytically active [71,202]. Several hydrolases have been confirmed to degrade polysorbates under formulation-relevant conditions, including CES1F, CES2C, LPLA2, PAF-AH, PPT1, and LIPA [54,106,131]. PLBL2, long considered active against polysorbate, was shown by knockout studies to lack polysorbate-degrading activity, although it frequently co-purifies with mAbs [200,237].

Despite extensive efforts to characterize polysorbate-degrading enzymes in Chinese hamster ovary (CHO) cell-derived biotherapeutics [106,131], the mechanisms underlying their persistence during downstream processing remain poorly understood. Previous studies showed co-elution driven by physicochemical similarity between HCPs and mAbs during chromatographic separation [115,130]. Other studies suggest that weak, reversible protein-protein interactions may enable certain HCPs to ‘hitchhike’ on therapeutic antibodies throughout purification [2,75,149]. This poses a significant challenge for downstream purification strategies, as hitchhiking behavior is highly product specific and difficult to predict.

Characterizing these interactions is technically challenging because they are typically low-affinity, transient, and occur at sub-ppm concentrations. Previous attempts using surface plasmon resonance (SPR) and microscale thermophoresis (MST) reported limited sensitivity and high variability, leaving the interaction landscape largely unexplored [75]. Mass spectrometry and oxidative labeling offered structural insights but lacks throughput for systematic screening [75]. Consequently, no broadly applicable platform exists for profiling HCP-mAb interactions.

Biolayer interferometry (BLI) is a label-free optical biosensing technique that enables real-time kinetic analysis with high sensitivity. It also supports the parallel screening of multiple interactions. While BLI has been widely applied in antibody discovery and epitope binning [7,155], its potential for detecting weak, reversible HCP-mAb interactions has, to the best of our knowledge, not been reported to date. This study addresses this gap by developing a BLI-based assay tailored to detect weak, reversible interactions between mAbs and polysorbate-degrading HCPs.

We systematically screened four representative polysorbate hydrolases (CES1F, LPLA2, PAF-AH, and PPT1) against eight model antibodies. To evaluate the relevance of these findings for downstream processing, we performed a Protein A chromatography experiment using PLBL2 as a model HCP. Our results provide the first systematic dataset linking

HCP-mAb interaction strength to downstream persistence and highlight the potential of quantitative interaction profiling for early risk assessment of biologics development.

5.2 Material and Methods

5.2.1 Model antibodies

In this study, eight different model antibodies were used (Table 5.1). All model antibodies were produced in-house by Boehringer Ingelheim Pharma GmbH & Co. KG. The theoretical isoelectric point (pI) of each antibody was calculated based on its amino acid sequence.

Table 5.1: Subtype and theoretical pI of model antibodies

Antibody	Subtype	pI
mAb-1	IgG1	9.1
mAb-2	IgG1	8.9
mAb-3	IgG4	7.8
mAb-4	IgG4	7.8
mAb-5	IgG1	9.6
mAb-6	IgG1	8.8
mAb-7	IgG1	9.2
mAb-8	IgG1	7.9

5.2.2 Expression and purification of recombinant HCPs

Hydrolases were recombinantly expressed using a transposase-based system. The coding sequences were derived from the CHO K1 transcriptome and cloned into a transposon-based expression plasmid. After transfection and selection, stable cell pools were cultivated, and recombinant proteins were purified from the harvested cell culture supernatant. Purification was performed using a three-step chromatographic protocol on an ÄKTATM Avant 25 system (Cytiva Life Sciences, Marlborough, USA), comprising immobilized metal affinity chromatography (HisTrapTM FF crude, Cytiva Life Sciences, Marlborough, USA), Strep-Tactin affinity chromatography (StrepTrapTM XT, Cytiva Life Sciences, Marlborough, USA) and size exclusion chromatography (HiLoad Superdex 200, Cytiva Life Sciences, Marlborough, USA) for final polishing. Monomeric fractions were pooled, aliquoted and stored at -70 °C. Further details on the expression and purification strategy have been described previously [131]. HCPs included in this study are listed in Table 5.2.

Table 5.2: HCPs selected for interaction studies, including pI, molecular weight (MW) and UniProt accession.

HCP	Abbreviation	Gene name	pI	MW (kDa)	Uniprot accession
Carboxylesterase 1f	CES1F	<i>Ces1f</i>	7.9	65	A0A061I7X9
Group XV phospholipase A2	LPLA2	<i>Pla2g15</i>	6.3	50	G3HKV9
Platelet-activating factor acetylhydrolase	PAF-AH	<i>Pla2g7</i>	8.6	52	A0A8C2M2A7
Palmitoyl-protein thioesterase	PPT1	<i>Ppt1</i>	8.0	37	G3HN89
Phospholipase B-Like 2	PLBL2	<i>Plbd2</i>	5.9	66	G3I6T1

5.2.3 Biotinylation of proteins

Ligand biotinylation was performed with EZ Link™ NHS PEG4 biotin (Thermo Fisher Scientific, Waltham, USA) to target solvent-accessible primary amines. A 20 mM stock solution was freshly prepared in deionized water and diluted to 0.2 mM for use. The proteins were adjusted to 1 mg/mL and the reagent was added to achieve a 3:1 molar coupling ratio (biotin:protein). The reaction was processed for 30 min with gentle mixing. Excess reagent was removed using Zeba™ 7K MWCO spin desalting columns (Thermo Fisher Scientific, Waltham, USA).

5.2.4 BLI based interaction assay development

Several Octet biosensor formats and assay orientations were initially evaluated. These included Fc-mediated antibody capture using Octet® ProtA and Octet® AHC Biosensors, as well as His-tag-based immobilization of recombinant HCPs on Octet® HIS2 Biosensors (Sartorius, Göttingen, Germany). However, these approaches were found unsuitable due to unstable baselines and high non-specific binding. The most robust configuration was achieved using biotinylated HCPs immobilized on Octet® High Precision Streptavidin (SAX) Biosensors (Sartorius, Göttingen, Germany), combined with monoclonal antibodies as analytes in solution.

Ligand loading scouts were performed for each biotinylated HCP to identify the lowest concentration ensuring stable immobilization and sufficient signal. A two-fold dilution series from 40 to 1.25 µg/mL was tested under standard assay conditions using a fixed analyte concentration. Sensorgrams were reviewed for stable loading without overshoot, minimal baseline drift, and clear association and dissociation phases.

All experiments were performed on an Octet® HTX system (Sartorius, Göttingen, Germany) in 384-well plates with 80 μ L per well. Data acquisition and analysis were conducted using Octet® BLI Discovery 13.0 and Octet® Analysis Studio 13.0 (Sartorius, Göttingen, Germany).

Prior to use, SAX biosensors were prehydrated for 1200 seconds in assay buffer before use. The optimized assay buffer consisted of 10 mM HEPES, 25 mM NaCl, 0.05% CHAPS, and 0.3% BSA at pH 7.0, selected to minimize non-specific binding while maintaining protein stability. Assays were conducted with orbital shaking at 1000 rpm.

The final assay protocol included five steps: baseline equilibration (180 seconds), ligand loading (600 seconds), second baseline (180 seconds), association (300 seconds), and dissociation (300 seconds). Biotinylated HCPs were immobilized at concentrations determined by prior loading scouts to ensure stable capture and reproducible signal. Each interaction was measured across a nine-point, two-fold analyte dilution series ranging from 2 to 533 nM. For each run, unloaded sensors were included to quantify non-specific binding, and ligand-loaded sensors held in buffer served as drift controls.

5.2.5 Quantitative binding analysis

Sensorgrams obtained from BLI experiments were preprocessed to correct for non-specific binding (NSB) and baseline drift. This included double referencing using unloaded sensors and buffer blanks, baseline alignment, and interstep correction between assay phases. Two different analytical approaches were applied to evaluate the interactions between HCPs and mAbs: 1) kinetic model fitting and 2) equilibrium response analysis.

Kinetic model fitting

For interactions with sufficient signal quality, a global 1:1 Langmuir binding model was fitted across multiple analyte concentrations using Octet Analysis Studio 13.0 (Sartorius, Göttingen, Germany). The association rate constant (k_a), dissociation rate constant (k_d), and equilibrium dissociation constant (K_D) were determined from the fits. Only those that met the following predefined quality criteria were included in the analysis: $R^2 > 0.95$, residuals within ± 10 % of the maximum response, and relative fitting errors $< 10\%$.

Equilibrium response analysis

For interactions where kinetic model fitting was not feasible due to low signal intensity or non-ideal sensorgram shapes, interaction strength was estimated using the initial slope (S_0) of the Langmuir binding isotherm. Equilibrium response values (R_{eq}) were calculated by averaging the signal over the final five seconds of the association phase across a nine-point analyte concentration series. These values were fitted to a 1:1 Langmuir isotherm, and the initial slope was derived by evaluating the first derivative of the binding curve at low analyte concentrations. The S_0 values were used to rank the relative interaction strength of all mAb and HCP combinations.

5.2.6 Validation of BLI screening via Protein A chromatography

To evaluate whether BLI-derived interaction profiles can predict the persistence of HCPs during downstream purification, a Protein A chromatography experiment was performed using PLBL2 as a model HCP. PLBL2 was chosen because it can be quantified using a commercially available ELISA [200]. In contrast, suitable immunoreagents for direct quantification are currently lacking for the polysorbate-degrading hydrolases investigated in this study [54].

PLBL2 was screened against all eight model mAbs using the same BLI assay format and evaluation strategy applied to polysorbate-degrading HCPs. Interaction strength was quantified by equilibrium response analysis and expressed as the initial slope (S_0) of the Langmuir binding isotherm. Based on these results, two antibodies representing high and low interaction profiles (mAb 2 and mAb 6, respectively) were selected for the chromatographic validation experiment.

Protein A chromatography was conducted on an ÄKTATM Avant 25 system using MabSelectTM Prisma resin (Cytiva Life Sciences, Marlborough, USA). Mock harvest material containing native PLBL2 was spiked with either mAb 2 or mAb 6 to achieve a load concentration of 5 mg/mL. The columns were equilibrated using 50 mM Tris-HCl buffer at pH 7.6, loaded at a residence time of 5 minutes, washed with three column volumes of equilibration buffer, and eluted with 50 mM sodium acetate buffer at pH 3.5. Fractions from the loading, washing, and elution steps were collected and neutralized immediately after elution.

PLBL2 concentrations in process fractions were quantified as previously described [216]. Total HCP clearance was confirmed using Cygnus 3G anti-CHO HCP antibodies (Southport, North Carolina, USA).

5.3 Results

5.3.1 Interaction screening of polysorbate degrading HCPs with monoclonal antibodies using BLI

To enable quantitative analysis of HCP-mAb interactions, a BLI-based assay was developed and optimized. The initial evaluation of biosensor formats revealed that Fc-mediated antibody immobilization (ProtA and AHC) and His-tag-based HCP capture (HIS2) were not suitable due to signal instability and high nonspecific binding (Supplemental Figure C1 and C2). The most robust configuration was achieved using biotinylated HCPs immobilized on streptavidin-coated biosensors, allowing sensitive detection of weak interactions.

This setup was used to systematically screen all combinations between four representative polysorbate degrading HCPs (CES1F, LPLA2, PAF-AH, PPT1) and eight monoclonal antibodies (mAb 1-8). Figure 5.1 shows representative sensorgrams for CES1F, illustrating typical association and dissociation behavior across the antibody panel. Most interactions

reached equilibrium during the association phase, with signal intensity increasing with analyte concentration and revealing a concentration-dependent specific interaction.

mAb 1, mAb 5, and mAb 7 exhibited the strongest response, while mAb 3 and mAb 6 showed minimal binding, and mAb 8 displayed intermediate signal levels. Comparable interaction patterns were observed for the remaining three polysorbate degrading HCPs (LPLA2, PAF-AH, and PPT1) across the same panel of monoclonal antibodies (Appendix Figure C4 – C6). For each mAb, the overall response trend remained consistent regardless of the HCP, suggesting that all four proteins exhibit a similar transient binding behavior toward the tested mAbs. Nevertheless, absolute differences in signal response were observed. CES1F yielded the highest response, followed by LPLA2, PAF-AH, and PPT1. When comparing sensorgrams, some interactions seem to be biphasic suggesting underlying rebinding effects or heterogeneous binding sites (Figure 5.1 D, E). Biphasic patterns are also seen when comparing the dissociation of mAb 1 to mAb 3 since the latter reveals an instant dissociation followed by a second slower dissociation phase (Figure 5.1 A, C). While some sensorgrams appeared biphasic, this may also reflect non-specific adsorption or analyte-analyte interactions at high concentrations. Thus, it is challenging to use a global 1:1 kinetic fitting model to truly describe all interactions.

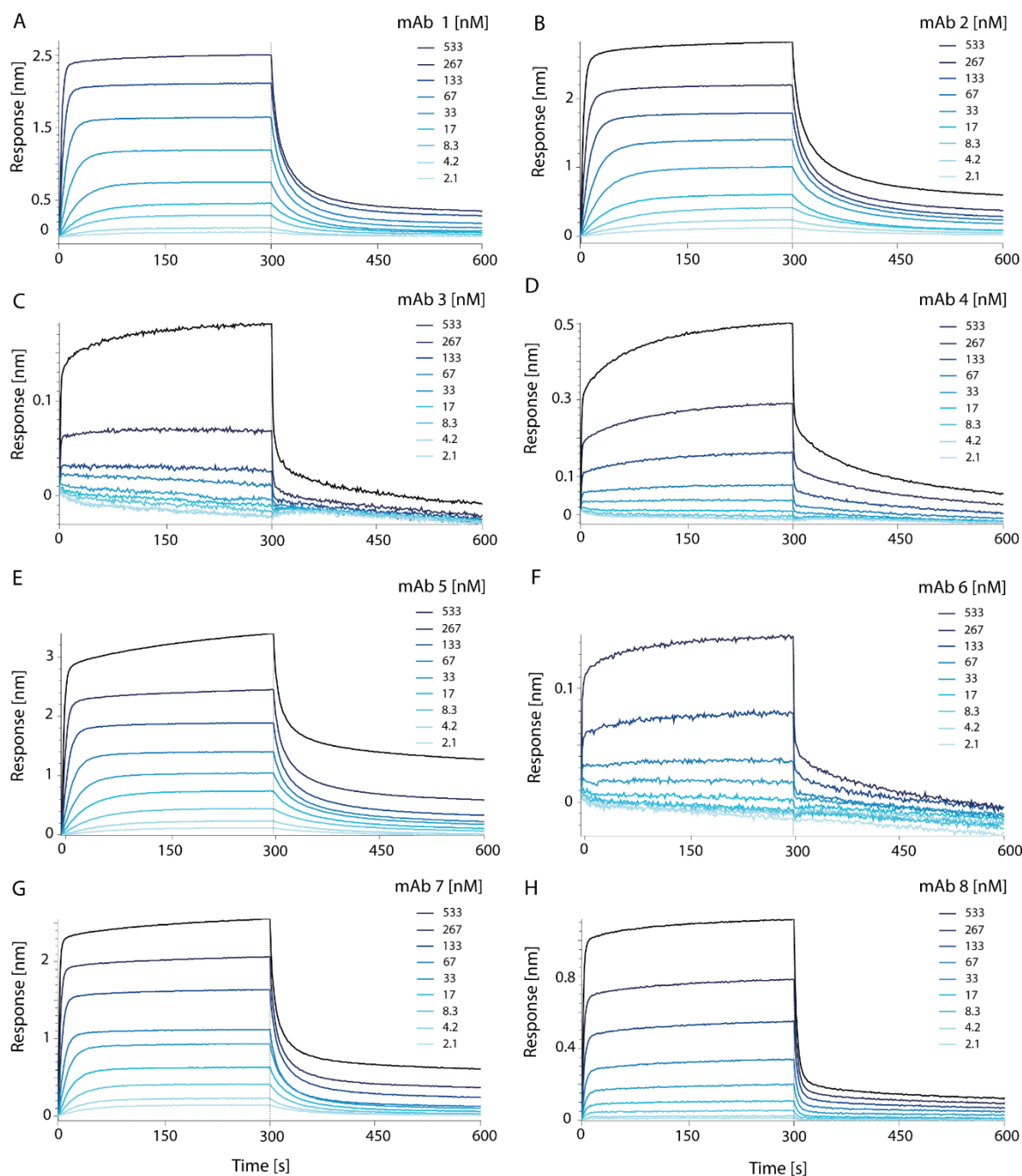


Figure 5.1: Representative BLI sensorgrams showing the interaction of CES1F with eight monoclonal antibodies. A: mAb 1; B: mAb 2; C: mAb 3; D: mAb 4; E: mAb 5; F: mAb 6; G: mAb 7; H: mAb 8. Antibody concentrations ranged from 2 to 533 nM, as indicated by the color legend. Sensorgrams displayed typical association and dissociation phases. Signal intensity increases with antibody concentration and varies between mAbs, reflecting differences in binding behavior while some show biphasic patterns.

5.3.2 Quantitative binding analysis

Two complementary analytical approaches were applied to characterize HCP-mAb interactions: (i) kinetic model fitting based on full association and dissociation phases, and (ii) equilibrium response analysis using steady-state binding levels. Both methods provide

affinity estimates and enable a comprehensive assessment of interaction strength. Prior to analysis, all sensorgrams were preprocessed to ensure data quality and comparability. This included double referencing to correct for nonspecific binding and baseline drift.

5.3.2.1 Kinetic model fitting

Kinetic parameters (k_a , k_d , K_D) were determined by global fitting of a 1:1 Langmuir model using Octet Analysis Studio 13.0 (Sartorius). The model simultaneously estimated the association rate constant (k_a), dissociation rate constant (k_d), and the maximum binding response (R_{max}), with the equilibrium dissociation constant (K_D) calculated as the ratio of k_d to k_a .

Only interaction profiles that met the predefined quality criteria were included in the analysis. These criteria included a clear signal increase during the association phase, a coefficient of determination (R_2) above 0.95, residuals within ± 10 % of the maximum response, and relative fitting errors below 10 %. Interactions that did not meet these standards were excluded from further evaluation.

Kinetic parameters were successfully determined for five of the eight mAbs across all four HCPs (Table 5.3). The resulting K_D values span approximately one order of magnitude, ranging from ~ 40 nM to ~ 400 nM. Most interactions showed K_D values between 40 and 90 nM. However, mAb 8 consistently showed higher K_D values, indicating weaker binding. CES1F and LPLA2 exhibited stronger affinities (lower K_D). PPT1 showed slightly weaker interactions.

Table 5.3: Kinetic parameters derived from global 1:1 Langmuir fits for HCP-mAb interactions that passed predefined quality criteria. For each interaction, the association rate constant (k_a), dissociation rate constant (k_d), and equilibrium dissociation constant (K_D) are reported. Values represent mean estimates across the tested concentration range. Interactions involving mAb 3, mAb 4 and mAb 6 did not meet quality requirements and are not included.

	mAb	K_D [nM]	k_a [1/Ms]	k_d [1/s]
CES1F	mAb 5	40.16	4.36×10^5	1.75×10^{-2}
	mAb 7	44.02	6.83×10^5	3.01×10^{-2}
	mAb 2	55.01	2.96×10^5	1.63×10^{-2}
	mAb 1	62.48	3.87×10^5	2.42×10^{-2}
	mAb 8	262.30	4.30×10^5	1.13×10^{-1}
PAF-AH	mAb 5	43.03	4.35×10^5	1.87×10^{-2}
	mAb 7	50.56	6.88×10^5	3.48×10^{-2}
	mAb 2	57.83	3.26×10^5	1.89×10^{-2}
	mAb 1	74.78	3.71×10^5	2.77×10^{-2}
	mAb 8	389.08	3.85×10^5	15.00×10^{-2}
PPT1	mAb 5	54.22	4.74×10^5	2.57×10^{-2}
	mAb 2	58.96	3.99×10^5	2.35×10^{-2}
	mAb 7	68.64	6.69×10^5	4.59×10^{-2}
	mAb 1	87.75	4.27×10^5	3.75×10^{-2}
	mAb 8	399.39	4.26×10^5	17.00×10^{-2}
LPLA2	mAb 2	44.75	3.23×10^5	1.44×10^{-2}
	mAb 5	47.51	3.47×10^5	1.65×10^{-2}
	mAb 7	49.01	6.81×10^5	3.34×10^{-2}
	mAb 1	61.86	3.45×10^5	2.14×10^{-2}
	mAb 8	372.80	3.33×10^5	12.40×10^{-2}

Across the datasets, association rate constants (k_a) were relatively consistent, typically falling within range of mid to upper hundred-thousands per molar per second. In contrast, dissociation rate constants (k_d) showed greater variability, ranging from low hundredths to nearly two-tenths per second. These observations suggest that the differences in equilibrium dissociation constants (K_D) are primarily driven by variations in complex stability

rather than association kinetics. Figure 5.2 further illustrates this trend, showing the dominant role of dissociation rates in determining overall affinity.

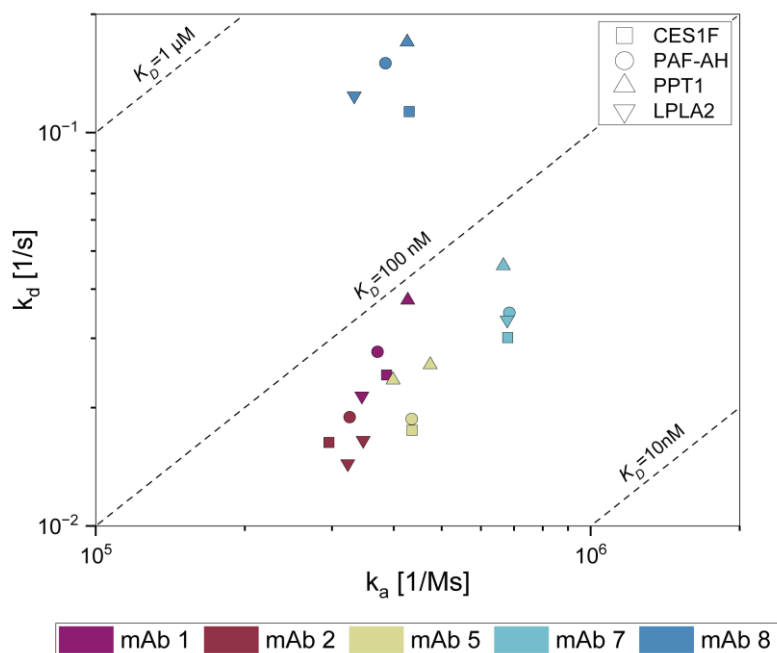


Figure 5.2: Scatterplot of association rate constant (k_a) versus dissociation rate constant (k_d) for all HCP-mAb interaction pairs that passed kinetic model fitting quality criteria. Colors indicate the mAb identity (mAb1-mAb8), and symbol shapes indicate the HCP (CES1F, LPLA2, PAF-AH, PPT1). Dashed iso- K_D lines (10 nM, 100 nM, 1 μ M) are included to visualize affinity levels. The plot illustrates that variation in K_D values across HCP-mAb pairs is primarily driven by differences in dissociation rates (k_d), while association rates (k_a) remain relatively stable.

5.3.2.2 Equilibrium response analysis

Equilibrium response analysis was performed to complement kinetic model fitting and to enable the analysis of low affinity interactions (Figure 5.3). Binding isotherms were generated from steady-state signals across the full analyte concentration range. These isotherms were then fitted to a 1:1 Langmuir model to estimate the apparent K_D values. Although most profiles demonstrated a good fit, saturation was not reached for all mAbs, which limited the reliability of the absolute K_D estimates and a comparison among each other.

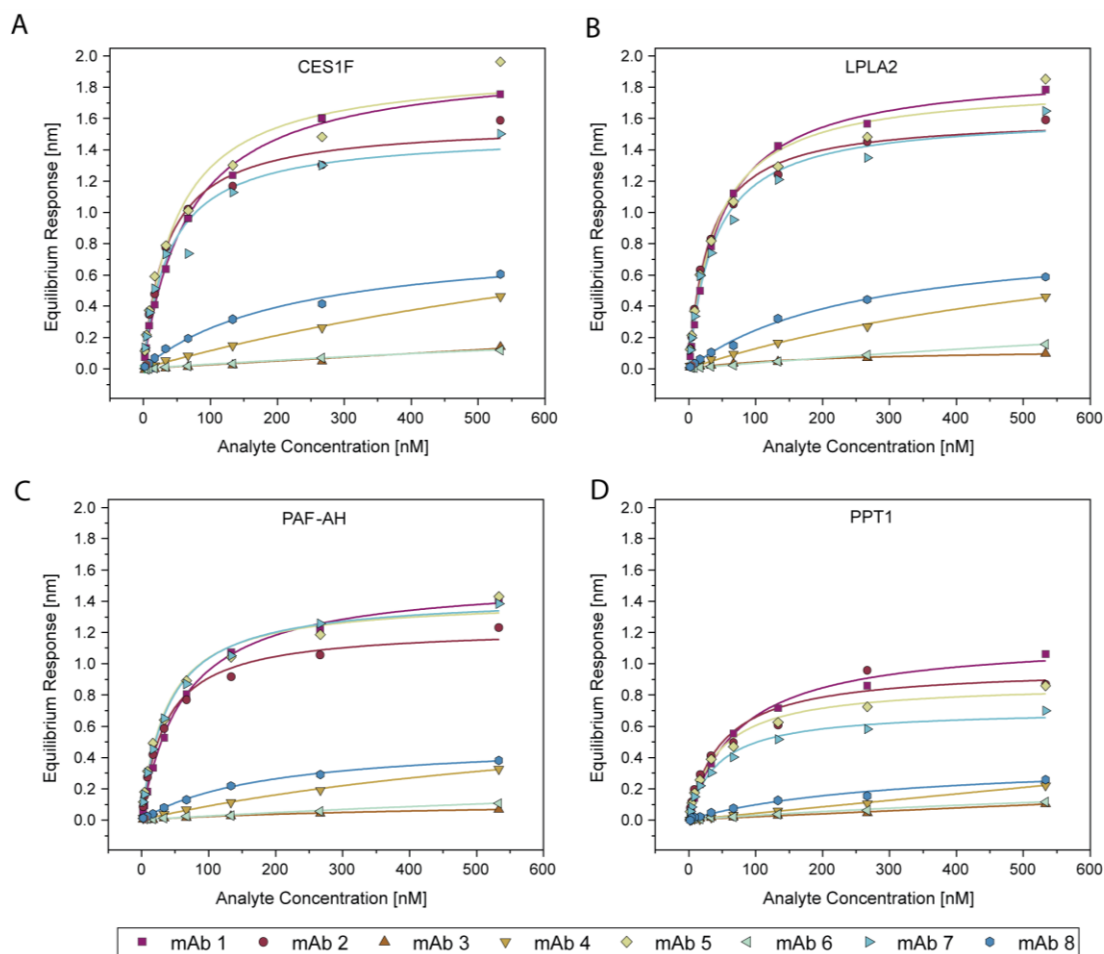


Figure 5.3: Steady-state binding curves of four polysorbate degrading HCPs measured across eight monoclonal antibodies (mAbs). For each HCP, equilibrium response values are plotted against increasing analyte concentrations (2 - 533 nM). Data points indicate experimental measurements, and solid lines represent Langmuir fits assuming a 1:1 binding model.

To overcome this, the initial slope (S_0) of the binding curve was used as an alternative measure of interaction strength. This approach ensured a quantitative evaluation of all HCP-mAb interactions, including profiles unsuitable for kinetic fitting. Table 4 summarized the initial slope (S_0) values across all HCP-mAb interactions.

Table 5.4: Initial slope values (S_0) derived from Langmuir fits for all HCP-mAb interaction pairs. S_0 reflects the ratio of maximum binding capacity ($R_{\max}$) to dissociation constant (K_D). Values span several orders of magnitude, enabling differentiation between strong and weak binders across the dataset.

	CES1F	LPLA2	PAF-AH	PPT1
	[nm/nM]	[nm/nM]	[nm/nM]	[nm/nM]
mAb 1	2.87×10^{-2}	3.99×10^{-2}	2.49×10^{-2}	1.54×10^{-2}
mAb 2	4.40×10^{-2}	5.31×10^{-2}	3.43×10^{-2}	2.01×10^{-2}
mAb 3	0.02×10^{-2}	0.06×10^{-2}	0.02×10^{-2}	0.02×10^{-2}
mAb 4	0.12×10^{-2}	0.14×10^{-2}	0.10×10^{-2}	0.04×10^{-2}
mAb 5	3.83×10^{-2}	4.41×10^{-2}	3.86×10^{-2}	1.94×10^{-2}
mAb 6	0.03×10^{-2}	0.04×10^{-2}	0.03×10^{-2}	0.03×10^{-2}
mAb 7	3.77×10^{-2}	4.26×10^{-2}	3.67×10^{-2}	1.64×10^{-2}
mAb 8	0.37×10^{-2}	0.34×10^{-2}	0.27×10^{-2}	0.13×10^{-2}

Kinetic model fitting was applied to interaction profiles that met stringent quality criteria, resulting in K_D values for five of the eight mAbs across all four HCPs. In contrast, equilibrium response analysis via S_0 enabled quantification for all mAb-HCP pairs, including those with low-affinity interactions or incomplete saturation, which were unsuitable for kinetic fitting. To quantify the agreement between the two methods, Spearman’s rank correlation coefficient was calculated [177]. The resulting correlation coefficient ($\rho = 0.9$) indicates a strong agreement between K_D and S_0 ranking, suggesting that equilibrium-based initial slope analysis reliably reflects affinity trends captured by kinetic modeling.

5.3.3 Comparative analysis across mAbs

For a comparative analysis of monoclonal antibody interactions across different polysorbate hydrolases, the initial slope of the binding isotherm (S_0) was used as a quantitative descriptor. Since each HCP ligand was immobilized at an individually optimized concentration, absolute S_0 values were not directly comparable. To enable relative comparison across the panel, S_0 values were normalized within each HCP, where 0 represents the weakest interaction and 1 indicates the strongest interaction. The resulting interaction matrix revealed consistent patterns across all HCPs. Ranking the antibodies by their median normalized interaction intensity revealed a clear distinction between those with low and high binding tendencies (Figure 5.4).

The highest interaction intensities were consistently observed for mAbs 2, 5 and 7, followed by mAb 1 with moderately strong binding. mAb 8 showed intermediate values, while mAbs 6, 3 and 4 exhibited the lowest interaction intensities across the dataset.

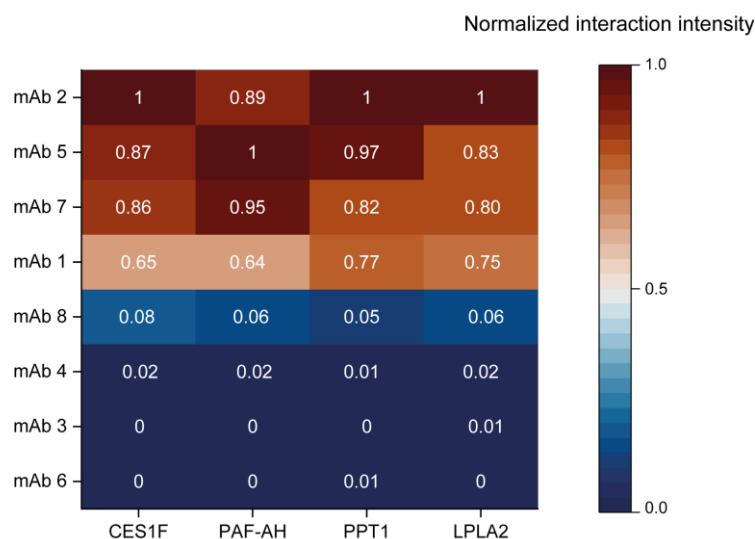


Figure 5.4: Normalized interaction intensities of monoclonal antibodies with polysorbate hydrolases. The heatmap illustrates the relative binding behavior of eight mAbs across four HCPs (CES1F, PAF-AH, PPT1, LPLA2). Interaction strength is expressed as normalized initial slope values (S_0), where 0 indicates the weakest and 1 the strongest interaction within each ligand. The color scale ranges from deep blue (weak interaction) to dark red (strong interaction), highlighting clear clustering of high-affinity antibodies.

To evaluate the contribution of electrostatic properties to interaction strength, the relationship between the theoretical isoelectric point (pI) and interaction strength was examined (Figure 5.5). Antibodies with higher pI values generally showed stronger interactions. However, two outliers (mAb 6 and mAb 2) showed the lowest and highest interactions despite having similar pI values. Under screening conditions, all mAbs carried a net positive charge (pI 7.9-9.6). Theoretical pI values among the polysorbate-degrading HCPs ranged from 6.3 (LPLA2) to 8.6 (PAF-AH), implying that their net charges at pH 7.0 may vary from slightly negative to moderately positive. Based on electrostatic considerations, the strongest attraction would thus be expected for mAb-LPLA2 pairs and the weakest for mAb-PAF-AH. Nevertheless, the observed ranking of mAb binding intensities remained highly consistent across all HCPs (Figure 5.4), indicating that factors beyond net charge contribute to the observed binding behavior.

Interpretation based on molecule format remains limited because the panel consists mainly of IgG1 antibodies and only two IgG4 antibodies. The two IgG4 molecules showed lower interaction intensities, however, this result cannot be generalized due to the limited representation of this subclass.

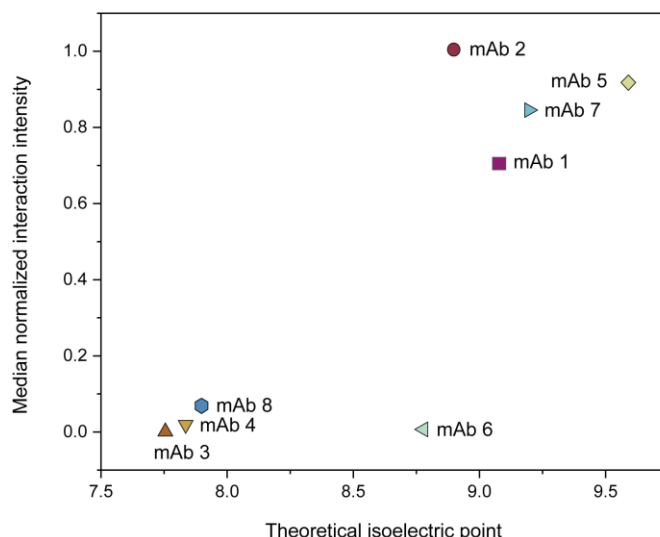


Figure 5.5: Relationship between theoretical pI and median normalized interaction intensity of mAbs. Each point represents one mAb. Interaction intensity was calculated as the median of normalized initial slope values (S_0) across four polysorbate hydrolases, where 0 indicates the weakest and 1 the strongest interaction. While antibodies with higher pI generally exhibited stronger interactions, outliers deviated from this trend, suggesting that factors beyond net charge contribute to binding behavior.

5.3.4 Validation of BLI based interaction screening using Protein A chromatography

A validation experiment was conducted to evaluate whether BLI-derived interaction profiles can predict HCP persistence during downstream purification. PLBL2 was used as a model HCP. Although PLBL2 is not enzymatically active against polysorbate, it was previously suspected of contributing to polysorbate degradation and remains of interest due to its potential immunogenicity [237]. Importantly, PLBL2 is one of the few CHO HCPs for which a commercially available, specific ELISA exists, enabling quantitative analysis across chromatographic fractions.

In contrast, the polysorbate degrading HCPs investigated in this study currently lack commercially available antibodies suitable for ELISA-based quantification [54]. Therefore, PLBL2 was selected as a representative model to validate whether interaction strength as determined by BLI correlates with impurity carryover in Protein A chromatography.

5.3.4.1 Interaction profiling of PLBL2

PLBL2 was screened against all eight model mAbs using the same assay format and evaluation strategy employed for the polysorbate degrading HCPs. Equilibrium response curves were recorded across a nine-point concentration series and analyzed using initial slope (S_0) analysis to quantify interaction intensity (Figure 5.6).

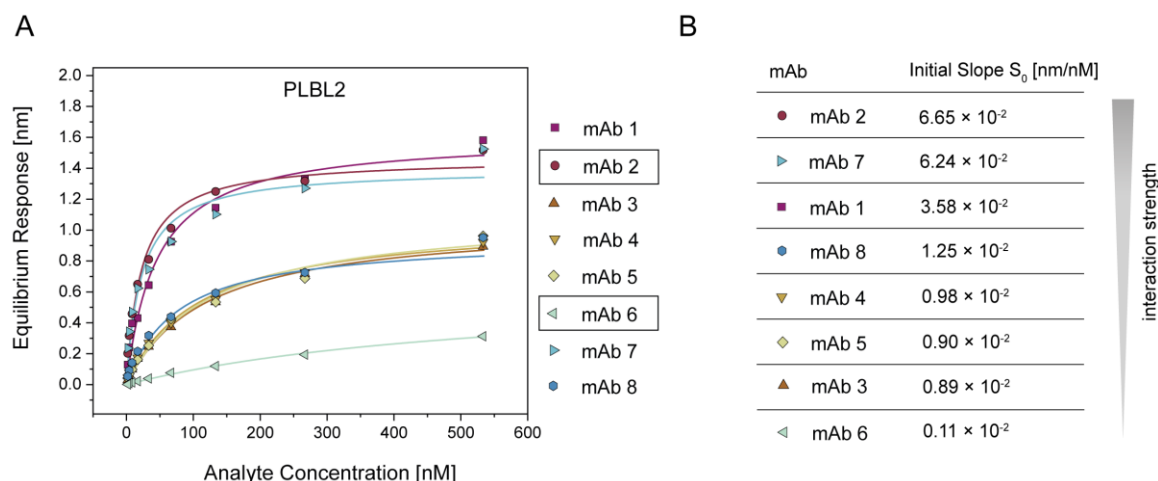


Figure 5.6: Interaction profiles of PLBL2 with eight monoclonal antibodies. A: Equilibrium response curves recorded across a nine-point analyte concentration series (2-533 nM) for PLBL2 interactions with different model mAbs. The curves show concentration dependent binding behavior, by varying signal intensities across the antibody panel. B: Initial slope values (S_0) derived from the equilibrium response data, providing a quantitative measure of interaction strength. The ranking reveals mAb 2 as the strongest binder, while mAb 6 shows the weakest interaction with PLBL2.

The resulting profiles showed a wider range of interaction strength compared to the other HCPs. While mAb 2 again showed the highest binding intensity, mAb 6 consistently exhibited the weakest interaction. Interestingly, the relative ranking of some antibodies shifted, e.g. mAb 5 and mAb 8. Based on these results, mAb 2 and mAb 6 were selected for downstream validation.

5.3.4.2 PLBL2 retention during Protein A chromatography

To test whether interaction strength predicts HCP retention, mAb 2 and mAb 6 were spiked into mock harvest material containing native PLBL2. Mock harvest material refers to clarified cell culture supernatant obtained from a non-producing CHO cell line, mimicking the matrix of a typical harvest but without the target monoclonal antibody. Then, the material was subjected to a standardized Protein A chromatography process. The load, wash and elution fractions were analyzed via ELISA.

Chromatography performance was comparable for both antibodies, with yields above 93 % and total HCP clearance close to 99 % (Table 5.5).

Table 5.5: Yield and overall HCP reduction during Protein A chromatography for mAb 2 and mAb 6.

Parameter	mAb 2	mAb 6
Yield [%]	93.7	94.5
Total HCP Reduction [%]	98.9	99.5

Despite similar levels of PLBL2 in the loading material, the eluate of mAb 2 contained approximately 40 ng/mL of PLBL2, while the eluate of mAb 6 only contained about 1 ng/mL (Figure 5.7A). Fractional analysis revealed that PLBL2 associated with mAb 6 was largely removed during the first wash. In contrast, PLBL2 bound to mAb 2 persisted across the wash and remained present in the eluate (Figure 5.7B). Overall, these results confirm that stronger BLI-derived interaction signals reflect a higher risk of HCP carryover during purification.

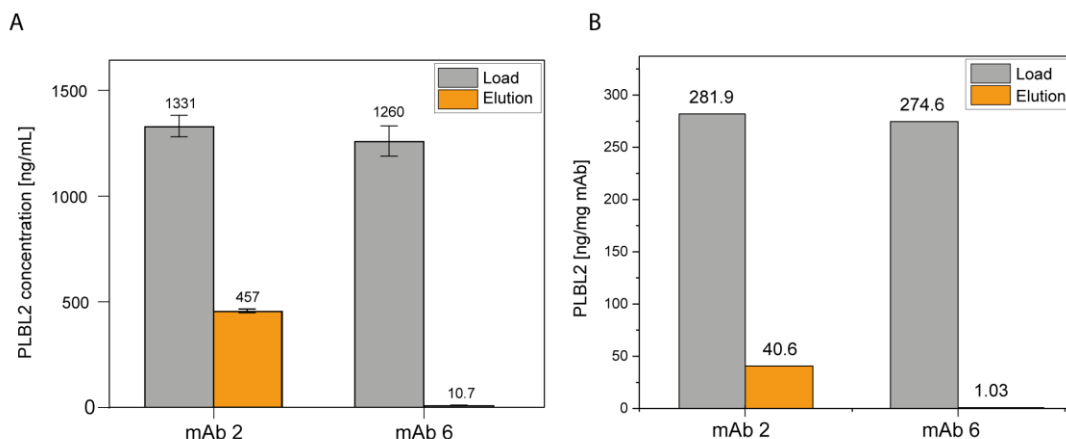


Figure 5.7: PLBL2 retention during Protein A chromatography for mAb 2 and mAb 6. A: PLBL2 concentration in load and elution fractions. B: PLBL2 levels normalized to antibody mass (ng PLBL2 per mg mAb). Both antibodies had comparable PLBL2 in the load, mAb 2 retained higher amounts in the elution compared to mAb 6.

5.4 Discussion

The interaction between polysorbate degrading HCPs and monoclonal antibodies represents a critical challenge in biologics development, particularly due to the risk of hitchhiking during downstream processing. While the enzymatic activity of polysorbate hydrolases has been extensively characterized, the extent to which weak, reversible protein-protein interactions contribute to their persistence during purification has remained unclear. This study provides a systematic analysis of weak, reversible HCP-mAb interactions and links them to potential hitchhiking behavior during downstream processing.

Using BLI, we observed consistent interaction profiles between several polysorbate hydrolases (CES1F, LPLA2, PAF-AH, PPT1) and a panel of monoclonal antibodies. These interactions were characterized by equilibrium dissociation constants (K_D) in the low nanomolar range, indicating high apparent affinity. However, the complexes exhibited rapid dissociation ($k_d > 0.01 \text{ s}^{-1}$), resulting in a transient binding mode with limited kinetic stability.

Although K_D values were derived using Langmuir fits, saturation was not reached in these assays, likely due to the high analyte concentrations required. In addition, the 1:1 fitting

model was not ideal for all mAb interactions as some clearly showed a biphasic behavior revealed by a two-phasic dissociation pattern. However, these heterogeneous binding phenomena may also arise from analyte-analyte interactions or non-specific adsorption at high concentrations, as previously reported for BLI assays [182]. To address this, equilibrium-based descriptors were evaluated as alternatives. The initial slope of the binding isotherm (S_0) correlated well with kinetic rankings and provided complete coverage of all mAbs. As S_0 is proportional to $R_{\max}/K_D$ under Langmuir assumptions, it reflects relative affinity even in the absence of saturation.

Compared to previous attempts using surface plasmon resonance (SPR) or microscale thermophoresis (MST), the BLI-based approach demonstrated superior sensitivity for weak, fast-dissociating complexes [75]. Hecht et al., 2022 previously described interactions between LPLA2 and antibodies as ‘ultra-low affinity’ and not detectable using SPR [75]. Similarly, microscale thermophoresis (MST) failed to detect binding for several enzymes, likely due to the rapid dissociation of these complexes. Our results show that under optimized conditions, BLI can resolve such transient interactions. Several methodological factors likely contribute to this improved sensitivity. First, the use of streptavidin-biotin immobilization on High Precision Streptavidin sensors provided a very stable surface and allows controlled ligand loading, which is essential for weak interaction where even small instabilities can distort the signal [5,76]. Second, immobilizing biotinylated HCPs and using intact mAbs as analytes increases the optical signal, whereas Fc-capture formats showed baseline drift and high nonspecific binding. Third, the dip-and-read format of BLI avoids fluidics and reduced mass transport limitations that can occur in microfluidic systems such as SPR [1,7], which might contribute to an improved detection of transient complexes.

The relevance of these findings for downstream processing was demonstrated using PLBL2 as a model HCP. We observed that differences in affinity measured by BLI translated into measurable differences in PLBL2 retention on the Protein A column. Given the transient nature of these interactions, one might expect that both antibody-PLBL2 complexes would be largely removed during the wash steps, resulting in similar clearance levels. Instead, the antibody with higher measured affinity showed higher PLBL2 carryover into the eluate. This indicates that affinity differences directly influence the extent of HCP persistence during the capture step. Our BLI experiments were performed with purified HCPs and mAbs, excluding potential co-purifying species such as DNA or high molecular weight antibody aggregates. The observed correlation between binding strength and PLBL2 persistence therefore supports direct HCP-mAb binding as a mechanism for hitchhiking. Nevertheless, other studies have shown that DNA-containing complexes or multicomponent aggregates can also promote HCP carryover [13,57,58,147]. Bhoyar et al. demonstrated that HCP persistence can result from the formation of HCP-antibody aggregates that co-elute during Protein A chromatography [13]. Similarly, Oh et al. described that HCPs can persist through co-aggregation with unfolded protein response components and chaperones [147]. In addition, co-elution of HCPs as functional protein networks has been proposed as an independent mechanism contributing to impurity persistence [125,151].

From a process development perspective, the ability to rank antibodies according to their tendency to bind polysorbate degrading HCPs can be a valuable tool for candidate selection. Antibodies with lower binding profiles may carry reduced risk of hitchhiking and associated polysorbate degradation in the final product. Analysis of approved mAbs therapeutics have shown that some HCPs are consistently present, highlighting the importance of product-specific interaction profiles [144]. Recent studies have shown that molecular descriptors such as charge distribution, hydrophobicity, and surface accessibility can predict chromatographic behavior of mAbs [78,128]. Extending these concepts to HCP-mAb interactions may allow quantitative-structure-property relationship (QSPR) models to identify antibodies with lower hitchhiking risk based on sequence or structural features. Integrating BLI-derived experimental data with in-silico predictions could thus enable early-stage risk assessment, supporting both molecule design and purification strategy development. The BLI-assay is also scalable and can be used in a high-throughput format, which makes it a practical tool for screening large numbers of antibodies.

Furthermore, this assay also offers opportunities for structural investigation of HCP-mAb interactions. Fragmentation studies, for example comparing intact mAbs with Fc and Fab fragments, could help localize interaction regions. Similarly, enzymatic deglycosylation of the Fc region could help to clarify if glycosylation patterns contribute to HCP binding, as suggested in previous studies [75].

Complementary approaches such as site-directed mutagenesis could further validate structural determinants of interaction [125]. Computational docking combined with electrostatic and hydrophobic surface mapping has proven effective for identifying potential interaction sites on mAbs and model proteins [161]. Integrating such tools with experimental BLI data could accelerate identification of high-risk regions and inform antibody design for reduced hitchhiking risk.

In summary, our study extends previous observations by directly quantifying interactions between polysorbate-degrading HCPs and mAbs, which were previously considered to be difficult to detect. The data demonstrates that relative affinity differences, even among transient complexes, can translate into measurable differences in HCP persistence during Protein A chromatography.

5.5 Conclusion

Our findings describe previous uncharacterized interactions between polysorbate-degrading HCPs and monoclonal antibodies and demonstrate their relevance for downstream processing. These interactions can significantly influence impurity clearance, as shown by differences in HCP retention during Protein A chromatography. The BLI-based approach developed here provides a practical and scalable method to detect and rank such interactions early in development, enabling informed candidate selection to mitigate polysorbate degradation in biopharmaceutical products.

Beyond screening, these insights open opportunities for predictive modeling and structural studies to identify molecular determinants of hitchhiking. Combining experimental interaction profiles with computational descriptors could support rational antibody design and optimized purification strategies.

Ultimately, integrating these concepts into process development can improve control over polysorbate degrading HCPs and enhance overall product quality.

Acknowledgements

We sincerely thank Joey Studts for his continued support of this work. We also thank Linus Weiss and Simon Fischer for valuable discussions and for providing hydrolase over-expression material and mock fermentations used in this study. Finally, we would like to thank Erik Machal for his careful review of the manuscript.

6 Specific polysorbate fingerprints of CHO hydrolases and implications for indirect assays

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Journal of Pharmaceutical Sciences (2025), doi: 10.1016/j.xphs.2025.104126

Abstract

Polysorbate degradation in biopharmaceutical formulations can impact the stability and efficacy of therapeutic proteins. This degradation is predominantly caused by specific residual host cell hydrolases present at sub-ppm concentrations. Their low abundance, combined with the lack of sensitive, enzyme specific detection methods, presents a major analytical challenge. This study presents a novel approach using reverse-phase ultra-performance liquid chromatography coupled with mass spectrometry (RP-UPLC-MS) to systematically analyse the specific polysorbate degradation patterns of hydrolases expressed by Chinese hamster ovary (CHO) cells. Our findings reveal distinct degradation fingerprints of five CHO-derived hydrolases, highlighting their unique preferences for different polysorbate species based on ester linkages (e.g. monoester or multiester), hydrophilic head groups and FA chain length. This study is the first of its kind to provide such detailed insights into the enzymatic cleavage patterns of polysorbates using enzymes directly derived from CHO cells.

Our results underscore the limitations of indirect polysorbate quantification assays, such as the fluorescence micelle assay (FMA) and 4-methylumbelliferone (MU4)-based hydrolytic activity assays, in accurately reflecting the activity of specific hydrolases. For instance, the FMA tends to overestimate the contribution of certain polysorbate species due to its reliance on micelle formation, while the MU4 assay's predictive reliability is limited by its use of surrogate substrates. In contrast, RP-UPLC-MS enables the precise identification of individual polysorbate species and their degradation products, providing a direct measure of hydrolase activity. The detailed enzymatic fingerprints obtained in this study pave the way for future advancements in enzyme classification and the potential application of computational tools for automated hydrolase identification.

Keywords: Excipient(s), Surfactant, Degradation product(s), Protein formulation(s), Enzyme(s), Monoclonal antibody(s), Drug product, Stability

6.1 Introduction

Polysorbate (PS) degradation by residual hydrolases poses a significant challenge in the biopharmaceutical industry [54]. Polysorbates, particularly Polysorbate 20 (PS20) and Polysorbate 80 (PS80), are non-ionic surfactants widely used in biopharmaceutical formulations. They stabilize proteins by preventing aggregation and surface adsorption [100,222]. Although PS plays a critical role as a protein stabilizer, some host cell proteins (HCPs) can hydrolytically degrade them [54].

The released free fatty acids (FFAs) are poorly soluble in water and after exceeding the solubility limits, fatty acid (FA) particles can form [45,67,172]. FA particle formation induced by enzymatic hydrolysis of PS is a subject of much debate in the field of biologics and represents a significant quality concern [48,99,102,172]. In addition to enzymatic hydrolysis, oxidative degradation of PS has also been identified as a critical degradation pathway, particularly under stress conditions such as the presence of iron and oxygen. Parenteral drug products are required to be essentially free of visible particles, with sub-visible particle specification limits set at 6000 particles per container for particles $\geq 10\text{ }\mu\text{m}$ and 600 particles per container for particles $\geq 25\text{ }\mu\text{m}$, as defined by the United States Pharmacopeia (USP <787>) and the European Pharmacopoeia (EP 2.9.19) [37].

Over the last decade, several HCPs have been identified as the primary cause of PS degradation [39,54,65,106,131,190]. Several CHO-derived hydrolases have been implicated in PS degradation. Frequently reported examples include Carboxylesterase 1F (CES1F), Carboxylesterase 2C (CES2C), Group XV phospholipase A2 (LPLA2), Platelet-Activating Factor Acetylhydrolase (PAF-AH), and Palmitoyl-Protein Thioesterase 1 (PPT1). These HCPs, which were classified as lipases and esterases, are present as impurities in the final drug products [65,71,117,140,238]. A variety of mitigation strategies have been presented to prevent PS hydrolysis [118,221], including the knockout and knockdown of lipases [32,217]. The addition of lipase inhibitors [169,236], optimized upstream and downstream development to reduce the amount of HCPs [112,118,130], or improved formulation conditions to prevent particle formation by shifting the solubility limits of the FFAs have also been proposed [169].

A major challenge in identifying and characterizing these hydrolases is their activity at extremely low concentrations ($< 1\text{ ppm}$) [71], which makes them difficult to detect by conventional analytical methods. Enzyme-linked immunosorbent assays (ELISAs) are widely regarded as the gold standard for the detection of total HCP content [157]. However, as ELISAs rely on immunoreactivity, they cannot detect non-immunoreactive proteins or provide information about individual HCPs [68,157,198]. Mass spectrometry can overcome this limitation by identifying individual proteins. However, its effectiveness is hampered by the wide range of protein concentrations in biopharmaceutical products, making their identification and quantification very challenging [18,50,208,239].

In addition, their concentration does not always correlate with their activity, as some hydrolases can aggregate or remain inactive [65,106,131,236,237]. Monitoring these

enzymes during downstream process development is highly desirable to develop targeted strategies for their removal [130]. However, this is often challenging due to the lack of commercial ELISAs for individual enzymes and the difficulty of implementing quantification for each critical HCP [54]. Previous studies have shown that different hydrolytic enzymes exhibit distinct PS degradation patterns [60,140]. PS20 is a complex mixture of ethoxylated sorbitan and isosorbide esters, that vary in their FA composition (Table 6.1) and the number of ester linkages (mono-, di-, multiesters). This structural diversity complicates their quantification and monitoring [120].

Table 6.1: Fatty acid composition of PS20 as listed in the European Pharmacopoeia. Adapted from Weber et al. 2023 [212].

Fatty acid		Percentage [%]
Lauric acid	C(12:0)	40-60
Myristic acid	C(14:0)	14-25
Palmitic acid	C(16:0)	7-15
Oleic acid	C(18:1)	≤ 11
Capric acid	C(10:0)	≤ 10
Caprylic acid	C(8:0)	≤ 10
Stearic acid	C(18:0)	≤ 7
Linoleic acid	C(18:2)	≤ 3
Caproic acid	C(6:0)	≤ 1

Traditional analytical methods, such as the fluorescence micelle assay (FMA) or hydrolytic activity assays using 4-methylumbelliferone (MU4)-based substrates, can provide a first indication of enzymatic activity towards polysorbates.

The FMA is a widely used indirect assay for the quantification of polysorbates [21,60,120]. The assay is based on the incorporation of a hydrophobic fluorescent dye, such as N-phenyl-1-naphthylamine (NPN), into micelles formed by surfactants like PS20 and PS80.40. The resulting fluorescence signal is directly proportional to the micelle concentration in solution, providing an estimate of the PS content. Although this assay can be applied in an automated and high-throughput setting, it does not distinguish between individual PS species and may lead to misleading results depending on the hydrolase’s specificity. For example, hydrolases with a preference for multiesters, may be overestimated based on the FMA signal because multiesters contribute more strongly to micelle formation [40,60,120].

Conversely, the MU4-based hydrolytic activity assay uses 4-methylumbelliferone esters to mimic the ester linkage in polysorbates. Upon hydrolysis, the release of 4-methylumbelliferone produces a measurable fluorescence signal [12,87]. By varying the chain length of the esterified FAs, this assay can provide insights into substrate specificity. However, as a surrogate assay, it may not always correlate with actual polysorbate degradation and may underestimate the activity of certain hydrolases [106].

Advanced techniques like reverse-phase high-performance liquid chromatography coupled with mass spectrometry (RP-UPLC-MS) allow for detailed analysis of polysorbate degradation patterns, or “fingerprints” [25,52,53]. These fingerprints provide a unique opportunity to identify specific hydrolases based on their degradation. Understanding these preferences is important as the hydrolase specific selectivity in PS degradation can influence the outcome of indirect assays like FMA [120].

This study systematically analyses the specific PS degradation patterns of hydrolases expressed by Chinese hamster ovary (CHO) cells. This includes investigating their preferences for different PS species and their impact on both indirect (FMA, MU4 based assay) and direct (RP-UPLC-MS) polysorbate assays.

6.2 Material and Methods

6.2.1 Materials

Polysorbate 20 high purity (PS20 HP) was obtained from Croda GmbH (Nettetal, Germany). PS hydrolases were produced in-house at Boehringer Ingelheim Pharma GmbH & Co. KG (Biberach an der Riss, Germany). Histidine and sodium chloride were purchased from Sigma-Aldrich (St. Louis, USA). Substrates and dyes for the activity assays were purchased from different suppliers: 4-methylumbelliferone from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), N-phenyl-1-naphthylamine (NPN) from Sigma-Aldrich (St. Louis, USA).

6.2.2 Expression and purification of recombinant hydrolases

The recombinant hydrolases were prepared in-house as described previously.¹³ Briefly, cell lines stably expressing the hydrolase were generated. Hydrolase sequences were extracted from the CHO K1 transcriptome and cloned into an expression plasmid containing a transposase recognition sequence and a C-terminal His- and Strep-tag. Stable hydrolase-expressing cell pools were cultivated for 5-12 days and then harvested by centrifugation. A two-step orthogonal capture using sequential His- and Strep-tag affinity chromatography, followed by preparative size exclusion chromatography was performed. Proteins were analysed by SDS-PAGE. Fractions containing the purified protein were aliquoted and stored at - 70 °C.

6.2.3 Reverse-phase ultra-performance liquid chromatography mass spectrometry (RP-UPLC-MS) and peak identity assignment

The RP-UPLC-MS method, adapted from Lippold et al. (2017) and Evers et al. (2020), employed an Ultimate 3000 UHPLC system (Thermo Fisher Scientific) coupled with an ACQUITY QDa mass detector (Waters Corporation) featuring an ESI (Electrospray ionisation) source [53,120]. Protein removal was achieved using a Waters Oasis®Max mixed-mode column (30 µm, 2.1 mm × 20 mm, 80 Å), while PS subspecies were separated using

a Poroshell 120 SB-C8 reversed-phase column (4.6×100 mm, $2.7 \mu\text{m}$; Agilent Technologies). The analytical gradient was achieved using three different solvents (100 % acetonitrile (solvent A), 100 % ultrapure water (solvent B), and 100 % methanol (solvent C)) at a flow rate of $0.7 \text{ mL}\cdot\text{min}^{-1}$. The gradient was initiated with 100 % solvent B for 4 minutes, followed by a linear increase to 36 % A and 64 % B at 6 minutes. Subsequently, the composition was adjusted to 71.2 % A, 5.0 % B, and 23.8 % C by 22 minutes and maintained until 35 minutes. At 35 minutes, the system was re-equilibrated to the initial conditions of 100 % B, which were held until the end of the 45-minute run.

Prior to MS detection 10 mM ammonium formate at a flow rate of $0.2 \text{ mL}\cdot\text{min}^{-1}$ was added by a T-piece to the mobile phase, to improve ionisation of the analyte. Analysis parameters included a 2 μL injection volume, column temperatures of 50°C for PS20, a 45-minute run time, and mass detection between 250-1250 Da in positive mode at 2 Hz sampling rate, with calibration standards ranging from 0.05 to $0.6 \text{ mg}\cdot\text{mL}^{-1}$. The measured m/z ratios, along with their corresponding charges, intensities, and retention times, were exported from Empower for further analysis using an algorithm [192]. The intensities of the m/z ratios of PS20 sub-species (e.g. POE sorbitan monolaurate (S12)) were subsequently assigned based on mass correspondence. Peak assignment was performed according to the method described previously [53].

6.2.4 Hydrolase activity assay using 4-methylumbelliferone-based substrates

Methylumbelliferone-based substrates are used to mimic the ester linkage in PS [12,54,60,65]. The hydrolytic activity of the respective purified hydrolases was measured using 4-methylumbelliferone-based esters. These are used as a surrogate substrate to mimic the ester bond in PS. Upon ester cleavage MU4 is released, leading to an increase in the fluorescence signal. By varying the length of the esterified carbon chain, different substrate specificities can be studied. Each substrate has MU4 esterified to a specific FA. Four MU4 esters were tested in this study: decanoic acid (C10), lauric acid (C12), palmitic acid (C16) and oleic acid (C18:1). For the preparation of the 300 μM substrate stock solutions, respective amounts of either MU4-C10, MU4-C12, MU4-C16 or MU4-C18:1 were dissolved in Dimethyl sulfoxide (DMSO). The final concentration of the MU4 substrate in the assay was 3 μM . Hydrolases were added to the reaction at a final concentration of $0.5 \mu\text{g} \cdot \text{mL}^{-1}$. Fluorescence was measured at 340 nm extinction and 450 nm emission wavelength with a microplate reader (SpectraMax M Molecular Devices, San José, USA). A dilution series of MU4 (0-3 μM MU4) was used for calibration to convert fluorescence increase to fluorophore release. The slope of released MU4 against time determined the release rate of the samples. The results were normalised with the used protein concentration. Negative buffer controls were included to exclude possible auto-hydrolysis of the substrate.

6.2.5 Monitoring polysorbate degradation via fluorescence micelle assay (FMA)

The hydrophobic fluorescent dye N-phenyl-1-naphtylamine (NPN) incorporates into micelles formed by surfactants such as PS20 and PS80, resulting in an enhanced fluorescence

6.3.2 Activity and cleavage specificity of CHO hydrolases towards polysorbate species

Previous studies have shown that different enzymes exhibit distinct specificities for PS esters [60,66,140]. In this study, five CHO-derived hydrolases were analyzed to investigate their preferences for different PS species. The hydrolases were recombinantly expressed and purified using sequential His- and Strep- tag affinity chromatography followed by size exclusion chromatography to achieve high purity [131]. Each enzyme was tested at a concentration of $20 \text{ ng}\cdot\text{mL}^{-1}$, which corresponds to approximately 1-0.1 ppm, assuming antibody formulation concentrations ranging from 20-150 $\text{g}\cdot\text{L}^{-1}$ [184,210]. Despite these low hydrolase concentrations, significant PS degradation occurred within a matter of days (Figure 6.2), underscoring the enzymatic activity of these hydrolases even at trace levels. In actual formulations, enzyme concentrations may be even lower. Such low enzyme levels typically remain undetected in conventional HCP ELISAs due to limited antibody sensitivity and specificity [19]. Similarly, their identification using mass spectrometry-based proteomic approaches is challenging because of the high dynamic range between abundant therapeutic antibodies and trace-level hydrolases [54].

Incubation of these enzymes with PS allowed the generation of specific degradation fingerprints that were analysed using RP-UPLC-MS. The hydrolysis of PS depends on several factors, e.g. the order of esters (mono-, di-, multiesters), the hydrophilic head group (sorbitan, isosorbide or polyoxyethylene), and the chain length of the esterified FA. Enzymatic treatment altered the distribution of the esterified subspecies, revealing hydrolase-specific preferences (Figure 6.2). These findings are consistent with previous studies that have demonstrated enzyme specific degradation patterns for PS, although previous work relied primarily on model hydrolases rather than those derived directly from CHO cells [60,66,140].

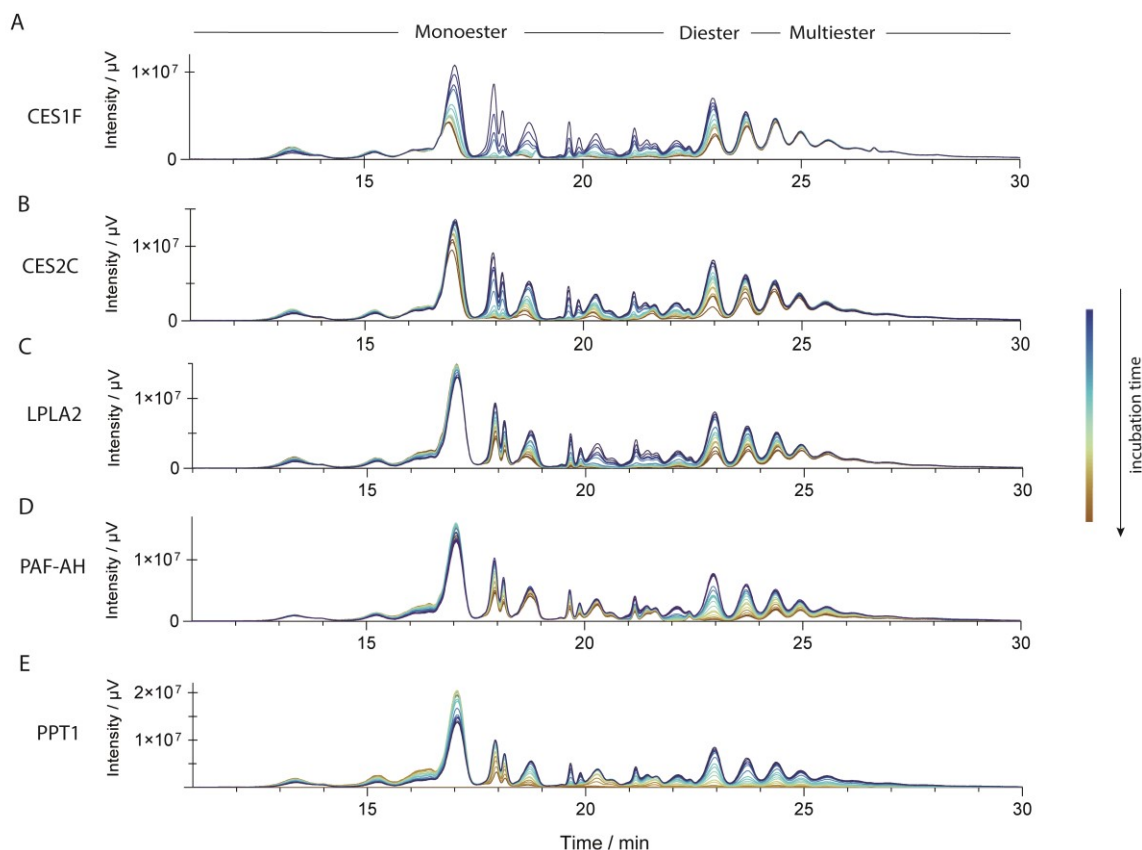


Figure 6.2: Total ion chromatogram (TIC) of PS20 following incubation with different CHO hydrolases. A: CES1F; B: CES2C; C: LPLA2; D: PAF-AH; E: PPT1. An initial PS20 HP concentration of $0.4 \text{ g}\cdot\text{L}^{-1}$ was used. Hydrolases were spiked into the formulation buffer at a concentration of $20 \text{ ng}\cdot\text{mL}^{-1}$. The samples were incubated at 25°C for varying time points ranging from 20 to 60 days, depending on the hydrolase activity. PS20 degradation was monitored using RP-UPLC-MS. The chromatograms reveal a hydrolase-dependent variation in peak intensity change, indicating specificity for different PS species.

CES1F showed broad specificity for monoesters across all head groups (Figure 6.2). The highest activity was observed against C12 monoesters – the dominant FA species in PS20. Despite its strong activity against monoesters, CES1F has no enzymatic specificity for multiesters. This suggests that its specificity is influenced by the structural properties of its substrate rather than abundance of the various PS species.

CES2C also displayed a preference for monoesters (Figure 6.2B). The peak intensities of the monoesters are clearly reduced over time, despite their continuous formation through the degradation of multiesters. Notably, isosorbide C12 and POE C12 monoesters were degraded faster than sorbitan C12 monoesters, indicating a lower specificity for sorbitan-based species. These results are consistent with previous observations that head group composition plays a critical role in determining enzyme specificity [140].

In contrast, LPLA2 showed the highest activity towards diesters, with a moderate reduction towards multiesters (Figure 6.2C). The relatively stable levels of sorbitan C12 monoesters suggest low specificity for these esters, a trend also observed by Glücklich et al.

for different model hydrolases [60]. PAF-AH showed a strong preference for multiesters, leaving monoesters largely intact (Figure 6.2D). PPT1 demonstrated the strongest preference for multiesters (Figure 6.2E). In particular, long-chain multiesters are rapidly degraded, leading to an increase in monoesters. This activity suggests that PPT1 prefers highly hydrophobic substrates.

These results show the unique degradation fingerprints of CHO hydrolases and their distinct preferences for PS ester species. A summary of the relative substrate preferences for mono-, di-, and multiesters is provided in Table 6.2. This variability provides a basis for understanding how enzymatic specificity affects the interpretation of PS degradation. While considering here a 2-dimensional separation, intensity over time, only a qualitative estimation of the degradation of the different PS20 HP species can be performed. Whereas a detailed, quantitative analysis of the sub-species is not possible.

Table 6.2: Overview of enzymatic preferences of CHO-derived hydrolases for different polysorbate ester types. Relative activity is estimated from RP-UPLC-MS degradation profiles (Figure 2): +++ = high, ++ = moderate, + = low, 0 = very low or negligible activity.

Enzyme	Monoester	Diester	Multiester
CES1F	++	+	0
CES2C	++	+	0
LPLA2	+	++	+
PAF-AH	0	++	++
PPT1	+	+++	+++

6.3.3 Influence of different CHO hydrolases on indirect PS20 HP degradation assays

Monitoring polysorbate degradation via fluorescence micelle assay

The fluorescence micelle assay (FMA) was used to investigate how the substrate preferences of CHO-derived hydrolases influence the PS concentration measured by this indirect method. Unlike RP-UPLC-MS, which directly quantifies individual PS species, the FMA estimates PS content based on the presence of micelles. This dependence introduces a bias towards PS sub-species with lower critical micelle concentration (CMC) that contribute more to micelle formation, such as diesters and multiesters, while underestimating monoester degradation [103]. To ensure comparability, all hydrolases were tested at the same concentration, and results from both methods were normalized to their respective blank controls.

CES1F showed low reduction in the FMA signal despite distinct monoester degradation observed by RP-UPLC-MS (Figure 6.3A). The decline in monoester concentrations over time was not reflected in the FMA due to the low contribution of monoesters to micelle formation [103].

CES2C caused a moderate reduction in PS concentrations as measured by FMA, whereas RP-UPLC-MS revealed a significant degradation of monoesters (Figure 6.3B). These results confirm that the FMA underestimates the activity of CES2C, consistent with its strong preference for monoesters.

LPLA2 caused a steady reduction in PS concentration as measured by both FMA and RP-UPLC-MS. Furthermore, the relatively uniform decrease in monoester and multiester concentrations suggests balanced activity across these species (Figure 6.3C). However, the activity profile in Figure 6.2C indicates a preference for diesters over multiesters. Similar to CES1F and CES2C, FMA underestimates the extent of LPLA2 mediated PS degradation compared to RP-UPLC-MS.

PAF-AH showed a weak, but gradual decrease in PS concentration over time, as shown by both FMA and RP-UPLC-MS. Figure 6.3D reveals a steady reduction in multiesters concentrations, while monoesters remain largely unaffected. The moderate decrease in the FMA signal correlated with the progressive loss of multiester species, which show lower CMC values compared to monoesters. However, the FMA overestimated the extent of PS degradation compared to RP-UPLC-MS because the assay primarily reflects changes in micelle-forming multiesters while failing to account for intact monoesters [120].

PPT1 caused the most pronounced reduction in PS content determined by FMA. RP-UPLC-MS data revealed nearly complete hydrolysis of multiesters after 5 days, followed by a gradual accumulation of monoesters reaching 80 % after 33 days (Figure 6.3E). While the FMA signal suggested almost complete degradation of PS, RP-UPLC-MS revealed that a significant proportion of monoesters remained intact, again demonstrating the FMA bias towards detecting multiester degradation.

The comparison between FMA and RP-UPLC-MS illustrates the discrepancies between these methods (Figure 6.3). While PPT1 treated samples showed a strong decline in FMA signal, RP-UPLC-MS revealed significant residual PS content. Conversely, the activity of CES1F against monoesters is severely underestimated in the FMA. This underscores the considerable limitation of the FMA: In cases where hydrolases like CES1F are present at low concentrations, PS degradation may remain entirely undetected. These findings highlight the need for careful interpretation of indirect PS quantification assays such as the FMA, especially in cases where hydrolase identity and specificity are unknown.

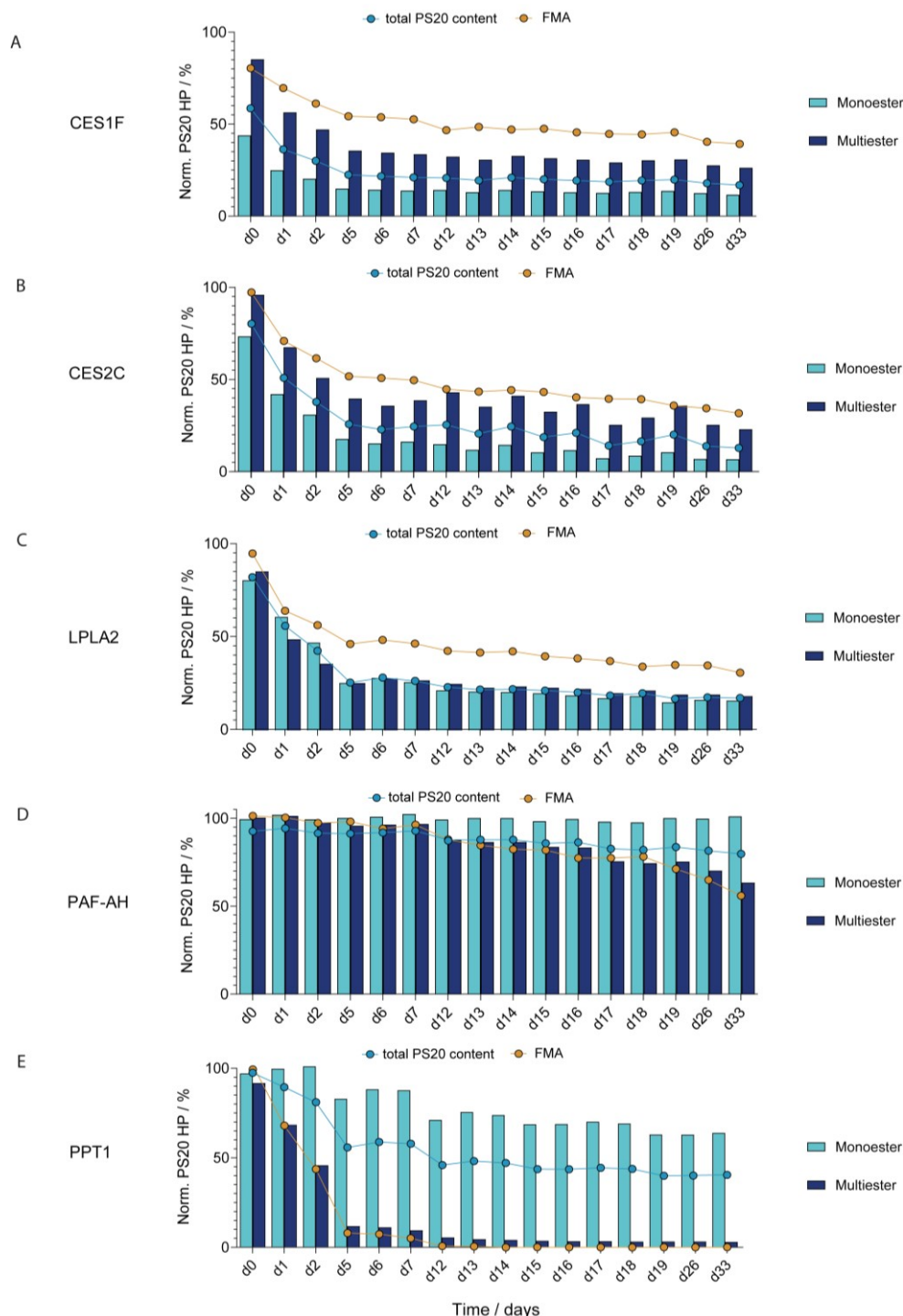


Figure 6.3: Comparison of polysorbate degradation measured by RP-UPLC-MS and FMA. Polysorbate degradation was assessed by spiking five CHO-derived hydrolases into formulation buffer at a concentration of $0.5 \mu\text{g}\cdot\text{mL}^{-1}$. A: CES1F, B: CES2C, C: LPLA2, D: PAF-AH, E: PPT1. The PS20 content was monitored over a period of 33 days. The orange circles represent polysorbate content measured by FMA, while the blue circles show total polysorbate content determined by RP-UPLC-MS. Bars indicate the degradation of specific polysorbate species as determined by RP-UPLC-MS, with monoesters shown in light blue and multiesters in dark blue. Data was normalized to the blank control.

6.3.3.1 Monitoring PS20 HP degradation using the MU4 based hydrolytic activity assay

The hydrolytic activity assay using 4-methylumbelliferone (MU4) esters can be used to estimate the hydrolytic activity in protein-containing samples.^{1,42} The reaction product MU4 can be detected by fluorescence emission. Typically, MU4 is esterified with a FA and serves as a surrogate substrate, mimicking the ester bond of PS. Given the different preferences of hydrolases, we investigated their activity for different substrates, here different MU4 esters. The following MU4 esters were used to investigate the influence of different carbon chain lengths on PS hydrolase activity: MU4 decanoate (MU-C10), MU4 laurate (MU-C12), MU4 palmitate (MU-C16) and MU4 oleate (MU-C18) (Figure 6.4).

CES1F and CES2C both showed moderate activity in the MU4 assay, with a shared preference for short-chain substrates (Figure 6.4 A and B). The highest activity for both enzymes was observed with MU4-C10, followed by moderate activity for MU4-C12 and MU4-C18. Activity against MU4-C16 was low. CES1F showed slightly lower overall activity compared to CES2C. LPLA2 showed the highest overall activity across all substrates, particularly for MU4-C10, MU4-C12 and MU4-C18 (Figure 6.4C). PPT1 showed strong activity for MU4-C18 and moderate activity for the other substrates tested (Figure 6.4 D). PAF-AH demonstrated the lowest overall activity in the MU4 assay, with activity rates for all substrates significantly lower than those of the other hydrolases (Figure 4E). While it showed slightly higher activity for C10 and C18 substrates, these rates were minimal compared to the activity of LPLA2. It is worth noting that lauric acid (C12) and oleic acid (C18) are particularly interesting as their esterified FAs are the main FAs of PS20 and PS80. For PS20 a ratio of 40-60 % of lauric acid is required, whereas oleic acid content of PS80 must be ≥ 58 % [136]. As different activity rates were obtained depending on the hydrolase and MU4 substrate, this may not always correlate with actual PS degradation. Figure 4F summarized the normalized PS20 concentration after a 1-day incubation with each hydrolase, as determined by RP-UPLC-MS. The actual degradation patterns reveal clear discrepancies compared to the MU4 assay results. For example, LPLA2 and PPT1 demonstrated high activity in the MU4 assay, but their PS degradation was found to be moderate to weak. On the other hand, CES1F exhibited the strongest PS degradation among all hydrolases, a result not directly reflected in the MU4 assay. These results highlight considerable limitations of the MU4 assay in forecasting PS degradation and careful interpretation of the data is required. The study by Kovner and co-workers further supports these findings, as discrepancies between PS20 degradation and the MU4-C8 substrate were observed for the hydrolases tested [106].

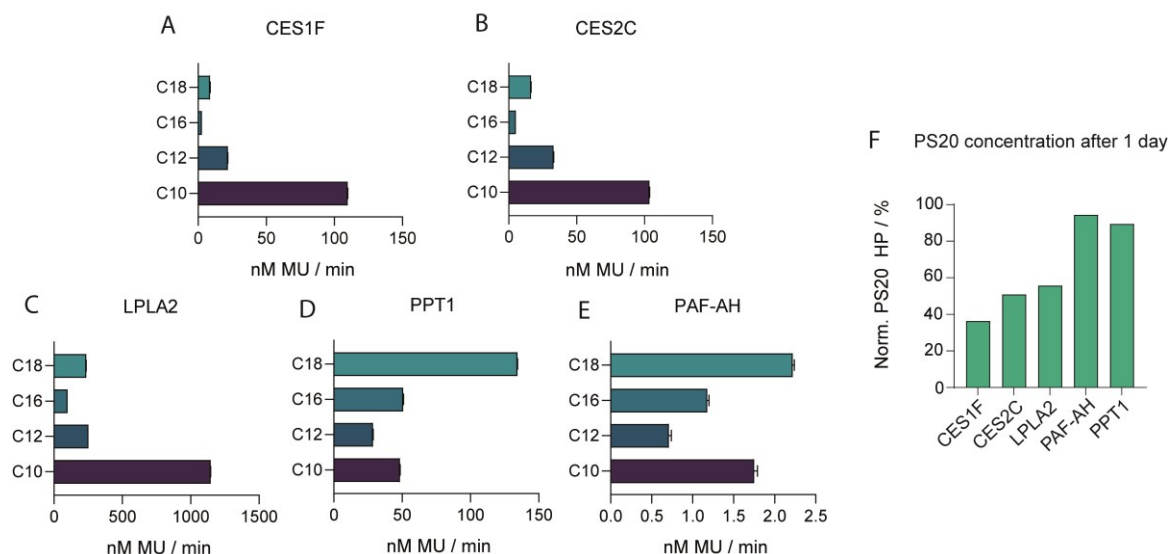


Figure 6.4: Hydrolase activity for different MU4 substrates compared to PS degradation. Subfigures A - E show the hydrolytic activity rates of CHO-derived hydrolases measured against four MU4 substrates (C10, C12, C16 and C18). A: CES1F, B: CES2C, C: LPLA2, D: PAF-AH, E: PPT1. Hydrolytic activity is expressed as the concentration of converted substrate (nM MU) per minute, with hydrolase concentrations standardized at $0.5 \mu\text{g}\cdot\text{mL}^{-1}$. Please note the different x-axis scales across the subfigures: Higher nM / min values indicate stronger enzymatic activity. Results are reported as the average of triplicates ($n=3$). Subfigure F illustrates the normalized PS20 HP concentration after a 1-day incubation with each hydrolase, as measured by RP-UPLC-MS. The 1-day timepoint was chosen because significant activity was already observed at this time for the applied hydrolase concentration. The comparison highlights discrepancies between MU4 activity and actual polysorbate degradation, underscoring the limitations of the surrogate assay in accurately representing enzymatic activity towards polysorbates in biopharmaceutical formulations.

Fluorescence-based activity measurements have been developed as high-throughput methods to provide rapid estimates of hydrolytic activity during biopharmaceutical process development. However, its reliance on surrogate substrates introduces biases that limit its predictive power. This discrepancy is particularly problematic for application of the assay in biopharmaceutical process development, where the MU4 assay may be used with the goal to identify hydrolases with high degradation potential. Hydrolases such as CES1F, which are highly active against polysorbates but poorly represented in the MU4 assay, may be overlooked. Conversely, hydrolases such as LPLA2, which show strong activity in the MU4 assay, may be overestimated in their contribution to PS degradation. While the MU4 assay has the advantage of speed and throughput, its limitations necessitate caution when interpreting results. These findings emphasize the need for complementary methods, such as RP-UPLC-MS, to ensure reliable characterization of relevant enzymatic activity.

6.3.4 Detailed cleavage patterns of CHO hydrolases using RP-UPLC-MS

To gain a detailed understanding of substrate specificity and cleavage patterns of CHO-derived hydrolases, RP-UPLC-MS analysis was used to follow the degradation of individual PS species over time. Therefore, an in-house developed generative machine learning model was used to extract the individual sub-species of PS20. The aim of this analysis was not only to confirm substrate preferences, but also to investigate the potential of such patterns to serve as enzymatic “fingerprints”. Normalized intensities (to initial sampling time point) of individual PS species, including sorbitan (S), isosorbide (I), and polyoxyethylene (POE) esters, were monitored at different time points. This detailed analysis revealed significant differences in substrate preferences, chain length specificity and head group affinities between the five tested hydrolases (Figure 6.5).

CES1F showed a distinct preference for monoester species, particularly those with medium FA chain length (Figure 5A). POE12, POE14, I12 and I14 showed the most pronounced decrease in normalized intensity, highlighting the strong activity of CES1F towards these substrates. In addition, the intensities of non-esterified species (S00, I00, POE00) increased over time, underlining its specificity for monoesters, as their hydrolysis leads to the generation of free head groups. In contrast, larger and more complex multiesters such as S12/12/12 remained largely intact.

CES2C exhibited a similar but slightly broader activity profile compared to CES1F (Figure 6.5B). Monoesters such as I12 and I14 were substantially degraded, with a concomitant increase in non-esterified species. However, CES2C showed lower activity against long chain sorbitan species such as S16 and S18. Notably, the I08 species increased over time with CES1F but decreased with CES2C. Interestingly, in contrast to CES1F, CES2C showed a slower degradation of the S12 species, which is the main component of PS20 HP [25].

LPLA2 displayed a strong preference for species with long FA chain length, particularly those containing C16 and C18 FAs (Figure 6.5C). The intensities of non-esterified species showed moderate increase over time, reflecting a balanced activity profile targeting both monoesters and multiesters.

PAF-AH exhibited a strong preference for I18:1 and S12/08, as shown by the most significant reductions in normalized intensity (Figure 6.5D). In addition, PAF-AH demonstrated notable activity against multiester species, further distinguishing its substrate specificity from other hydrolases.

PPT1 exhibits broad activity, particularly against diesters and multiesters. Among all hydrolases tested, this enzyme demonstrated the highest specificity for multiesters as evidenced by the reduction in normalized intensities of S12/12/12, S12/12/14, and S12/12/16 (Figure 6.5E). This strong preference for multiesters is accompanied by significant degradation of diesters such as S12/14 and certain monoesters including I14 and POE14. Depending on the enzyme and its preference for ester species, the hydrolysis susceptibility of different PS ester fractions could also influence the stabilizing properties of PS [40,66,199].

These findings further demonstrate that the activity is strongly influenced by both the ester chain length and the head group chemistry. Additionally, the resulting accumulation of free FAs can vary greatly depending on the hydrolase involved. Long-chain FAs such as palmitic acid (C16) and stearic acid (C18:0), specifically released by hydrolases such as LPLA2 and PPT1, possess lower aqueous solubility and thus a higher risk for particle formation [45,46,67].

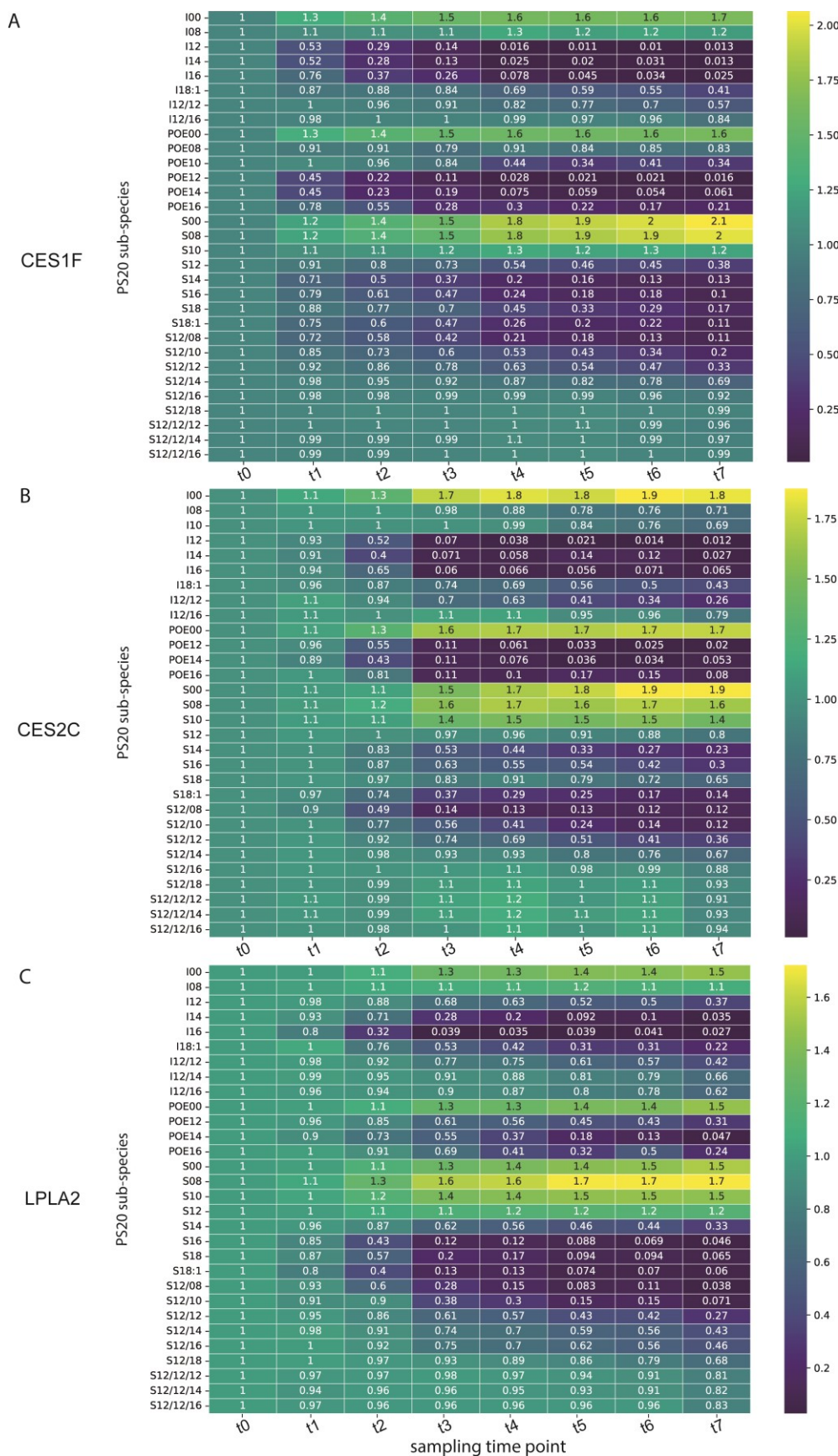


Figure 6.5: Normalized intensities for each polysorbate species at different timepoints. A: CES1F; B: CES2C; C: LPLA2; D: PAF-AH; E: PPT1. The labels S, I and POE represent

sorbitan, isosorbide and polyoxyethylene, respectively. The number indicates the chain length of the esterified fatty acid. Polysorbate degradation was assessed by spiking the respective purified hydrolase into formulation buffer. The formulation buffer contained 0.4 g·L⁻¹ PS20 HP, and the enzyme concentration was adjusted to 20 ng·mL⁻¹. Samples were incubated at 25 °C for varying time points ranging from 20 to 60 days, depending on the hydrolase activity.

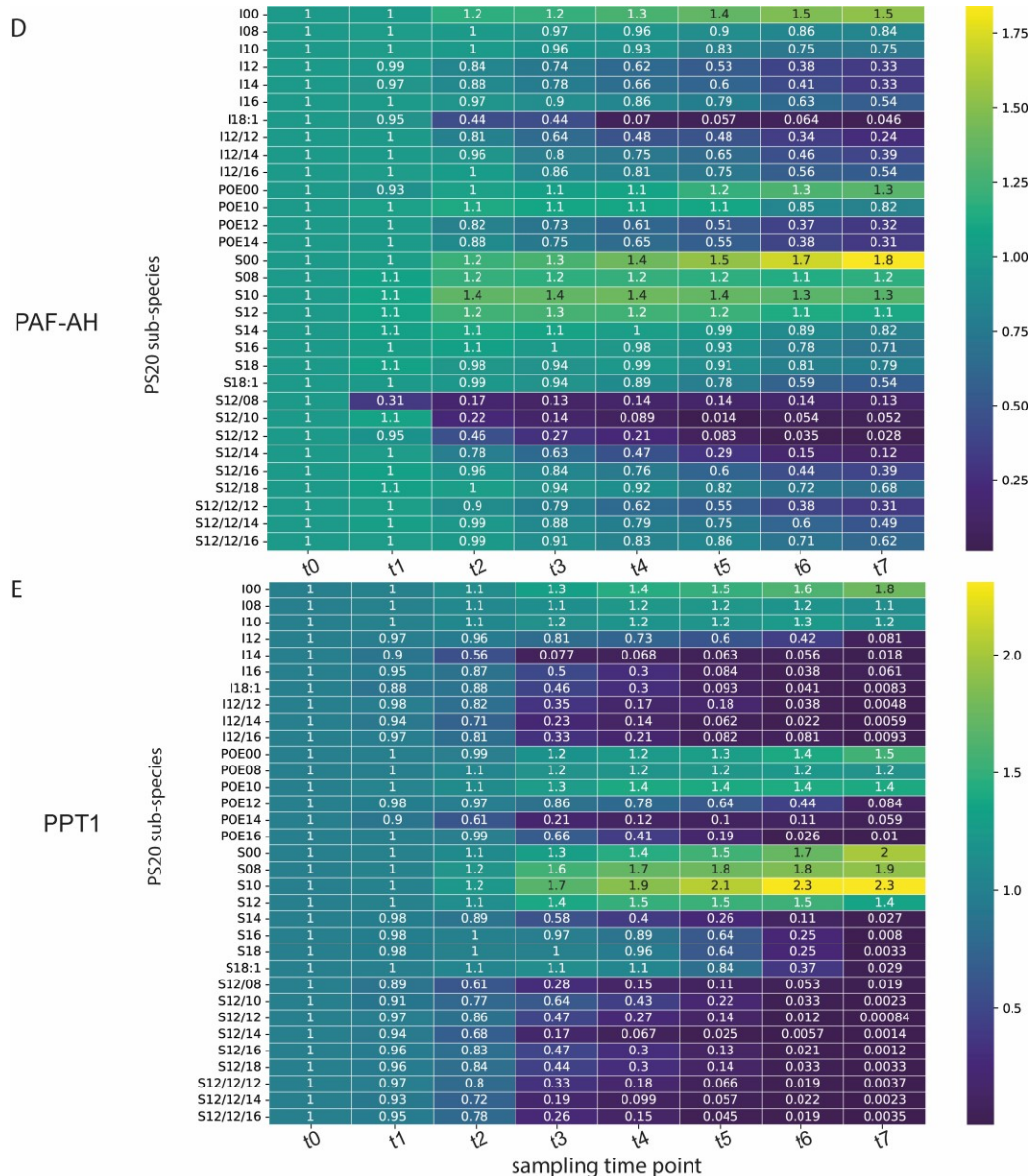


Figure 6.5: (continued)

Beyond the enzymatic release of such degradation products, the structural diversity of PS subspecies itself also contributes to formulation stability. Monoesters are generally considered less critical due to their higher solubility and limited contribution to micelle formation. In contrast, multiesters play a central role in interfacial stabilization. Their preferential degradation may compromise micelle integrity, thereby potentially accelerating particle formation. However, recent findings suggest that PS fractions rich in monoesters may offer

superior interfacial protection under dynamic stress conditions. For example, it has been shown that protein stabilization at interfaces is improved in formulations containing higher monoester content [40,199]. These observations underscore that not only the extent but also the pattern of PS degradation determines the risk to product quality.

This study provides a detailed analysis of PS degradation patterns for the five most active CHO-derived hydrolases out of 12 previously tested enzymes [131], revealing distinct degradation patterns that can serve as enzymatic fingerprints. This demonstrates the potential to classify hydrolases based on their unique enzymatic degradation patterns. By comparing the degradation profiles observed in antibody formulations with these fingerprints, it may be possible to identify the hydrolase responsible for PS degradation. This is particularly valuable for the development of targeted mitigation strategies, especially since polysorbate degradation often occurs without clear knowledge of the specific enzyme involved. Given the low concentrations of these hydrolases in biopharmaceutical formulations, such identification is challenging. In addition, while proteomic methods such as mass spectrometry can identify hydrolases, they do not always confirm enzymatic activity. Known cleavage patterns can provide important information about which enzymes are active or help exclude others. This insight is valuable for troubleshooting activities and optimizing process robustness during therapeutic development.

6.4 Conclusion

To our knowledge, this is the first study to analyze the PS degradation patterns of relevant CHO-derived hydrolases in such detail. While previous studies have employed methods such as UPLC-CAD to examine PS degradation, these approaches often suffer from co-elution of PS species, preventing the identification of unique enzymatic cutting patterns.¹² By utilizing RP-UPLC-MS and the automated extraction of individual sub-species via generative machine learning modelling we achieved a resolution that allows the precise monitoring of each PS species over time, revealing distinct substrate preferences and enzymatic fingerprints for each hydrolase.

The study also highlights the critical limitations of indirect PS assays, such as the FMA and MU4-based activity assays, in accurately reflecting enzymatic activity. These assays often incorrectly estimate the contribution of specific hydrolases due to their reliance on surrogate markers or indirect measurement principles. These findings highlight the need for complementary direct measurement techniques to gain a more accurate understanding of enzymatic activity.

Distinct enzymatic cleavage patterns offer a powerful approach to identify hydrolases responsible for PS degradation in biopharmaceutical formulations, thus enabling targeted mitigation strategies despite miniscule enzyme concentrations. These PS degradation fingerprints can lay the foundation for automated hydrolase classification using machine learning. While such an approach is beyond the scope of this study, our findings demonstrate the feasibility of this concept by showing how distinctly different hydrolases interact

with PS species. These findings provide a starting point for future work aimed at improving the stability of biopharmaceutical formulations through enzyme identification and targeted mitigation strategies.

Acknowledgements

The authors would like to thank Selina Nalbantoglu and Kristina Wösle for automation support and helpful discussions as well as Holger Thie for project management support.

7 A machine learning approach to identify active polysorbate degrading hydrolases in biopharmaceutical formulations

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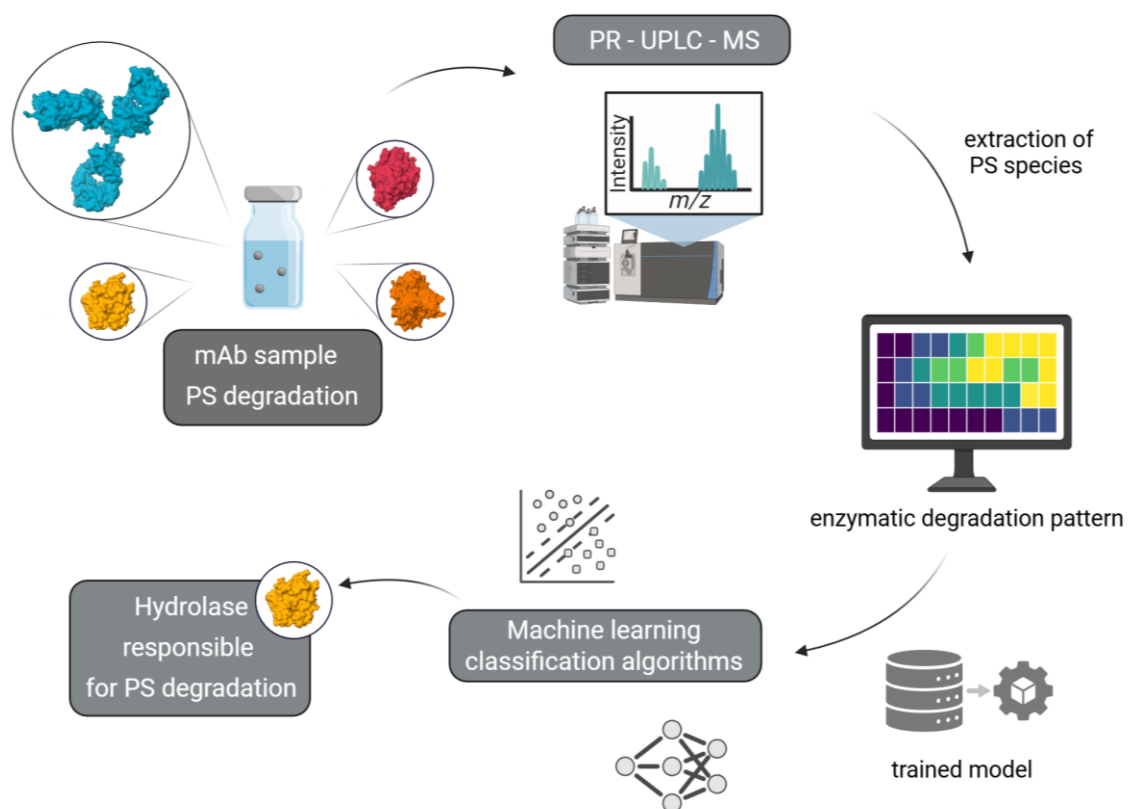
Under Review in: Journal of Pharmaceutical Sciences (2026)

Abstract

Polysorbate degradation by host cell-derived hydrolases presents a critical challenge in biopharmaceutical formulations. It can lead to fatty acid release, particle formation and reduced product stability. Mass spectrometry-based host cell protein (HCP) analysis is widely used for HCP identification, but detection becomes challenging in formulations where monoclonal antibodies are present in large excess. In such cases, hydrolases can remain undetected, despite being enzymatically active at trace levels.

In this study, we demonstrate that individual CHO-derived hydrolases generate distinct polysorbate degradation fingerprints, which can be detected by reverse phase ultra performance liquid chromatography coupled to mass spectrometry (RP-UPLC-MS) and classified using supervised machine learning. Models were trained on single-timepoint fingerprints comprising approximately 50 measurements for five hydrolases (CES1F, CES2C, LPLA2, PPT1 and PAF-AH). Evaluated algorithms included Logistic Regression, Random Forest, Gradient Boosting, Support Vector Classifier, Ada Boost, and Artificial Neural Networks. Seven out of eight models achieved 100 % accuracy on the test set, confirming that enzyme-specific information is preserved in single measurements in the presence of individual enzymes, independent of enzyme concentration or degradation time.

External validation using an independently prepared hydrolase spike sample confirmed the robustness of the models. Prediction confidence was high at early degradation stages and decreased at late stages, as enzyme-specific degradation fingerprints became more similar. This work presents an activity-based classification framework for the functional identification of polysorbate degrading hydrolases. The approach supports downstream monitoring and risk-based mitigation strategies by identifying the enzymes that drive polysorbate hydrolysis under formulation conditions.



Graphical Abstract created using BioRender.com

Keywords: Polysorbate degradation, hydrolases, host cell proteins, CHO, RP-UPLC-MS, machine learning, classification models, biopharmaceutical formulations, degradation fingerprints

7.1 Introduction

Polysorbates, particularly polysorbate 20 (PS20) and polysorbate 80 (PS80), are widely used as non-ionic surfactants in monoclonal antibody formulations [92,100]. Their primary function is to stabilize therapeutic proteins in the final drug product by preventing interfacial stress, aggregation, and particle formation [188]. Polysorbates are chemically highly heterogeneous mixtures consisting of a sorbitan or isosorbide core, polyoxyethylene (POE) chains and esterified fatty acids, resulting in hundreds of distinct molecular species [25,53].

Despite their stabilizing properties, polysorbates are susceptible to chemical and enzymatic degradation [48,49,102,107,179,213]. Of particular concern is the hydrolysis of polysorbates by host cell proteins (HCPs), which can lead to the release of free fatty acids and the formation of visible and subvisible particles [67]. Notably, enzymatic degradation can occur even at trace concentrations, highlighting the need for sensitive detection methods [54,71].

The functional identification of hydrolases in complex formulations remains a significant analytical challenge [54]. Mass spectrometry (MS)-based proteomics is widely used for HCP profiling in biopharmaceutical development. However, its application to formulated drug products is limited. High antibody concentrations (> 100 g/L) dominate the MS signal and can leave low-abundance impurities undetected. Furthermore, not all HCPs are enzymatically active under formulation conditions, and only a small subset contributes to polysorbate degradation [65,106,131]. This discrepancy between enzyme abundance and functional activity further complicates risk assessment and targeted mitigation.

Identifying which specific hydrolases are responsible for polysorbate degradation is therefore critical for risk-based process development. Such knowledge can guide interventions during upstream and downstream processing, for example by implementing targeted gene knockouts in production cell lines or by targeted optimization of purification steps [33,130,216,217].

Previous studies have shown that individual hydrolases exhibit distinct substrate preferences, resulting in characteristic degradation patterns or ‘fingerprints’ [60,129]. These fingerprints reflect the enzyme’s specificity towards different polysorbate species and can be captured using reverse phase ultra performance liquid chromatography coupled to mass spectrometry (RP-UPLC-MS). Because these fingerprints are highly complex and contain a lot of co-elution of PS species, manual integration is not feasible. An in-house developed generative model was employed to analyze the MS fingerprints and extract the intensities of the individual PS species [168]. Since there are approximately 35 PS species extracted, manual interpretation is cumbersome and not reliable. To address this challenge, we applied established classification algorithms such as Logistic Regression (logReg), Random Forest classifier (RFC), and Artificial Neural Networks (ANNs) to differentiate hydrolases based on their degradation fingerprints. Machine learning algorithms have been successfully applied in biopharmaceutical analytics for example to predict product quality from LC-MS data, interpret UV-based measurements for process monitoring, sub-visible particle classification or to monitor if a process is running under normal or critical conditions

[165,166]. Their ability to detect subtle, multidimensional patterns makes them suitable for differentiating hydrolases based on degradation fingerprints.

In this work, we demonstrate an activity-based classification approach that combines RP-UPLC-MS fingerprinting with supervised machine learning algorithms to identify polysorbate degrading hydrolases from single measurements.

7.2 Material and Methods

An overview of the experimental and computational workflow from enzymatic degradation to model validation is illustrated in Figure 1. It comprises (i) enzymatic degradation of polysorbate-containing formulations, (ii) acquisition of degradation fingerprints by RP-UPLC-MS, (iii) data preprocessing and normalization, and (iv) supervised machine learning for hydrolase classification. The following sections describe each step in detail.

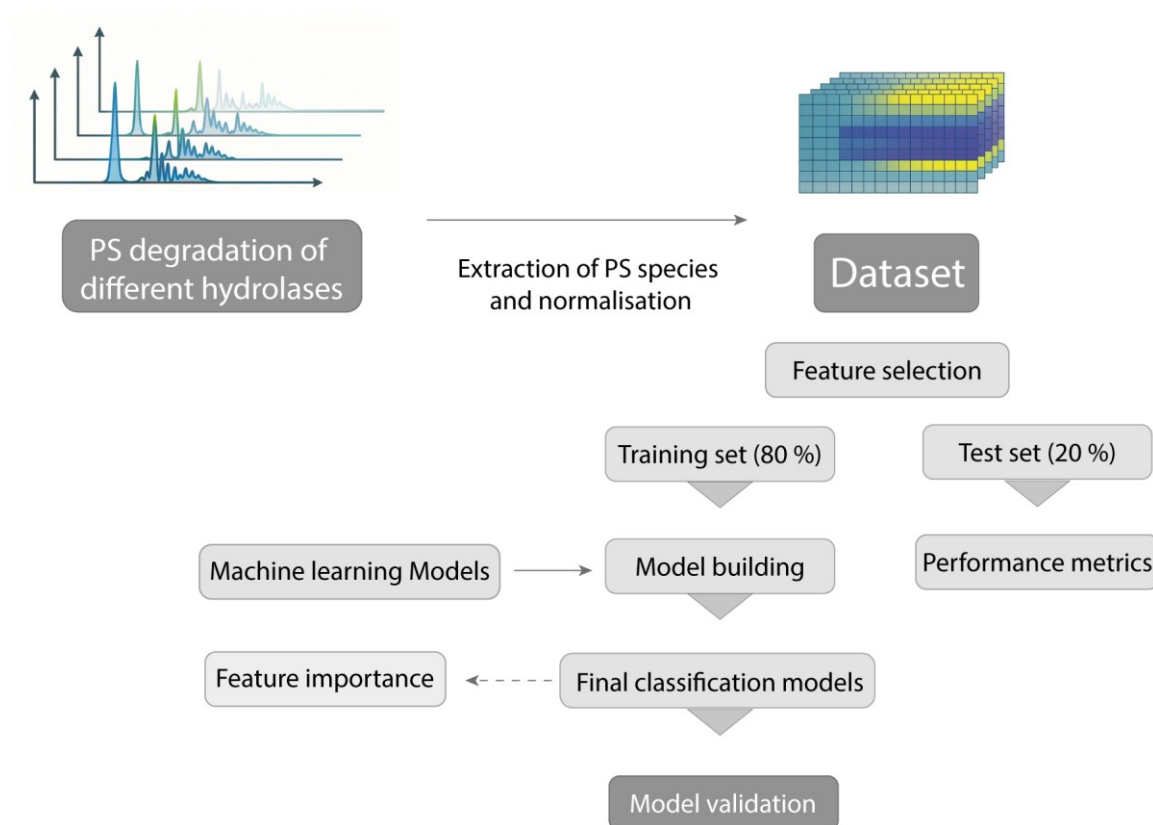


Figure 7.1: Overview of the experimental and computational workflow. CHO-derived hydrolases were incubated with polysorbate 20 HP containing formulations and analyzed by RP-UPLC-MS over hydrolase-specific time courses. Species were extracted via generative modeling, and intensities were normalized to the initial timepoint and compiled into a fingerprint dataset comprising five hydrolases, each with up to 50 timepoints and 23 polysorbate species. The dataset was split into training (80 %) and test sets (20 %), and different classifiers were trained. Final models were applied to external validation samples.

7.2.1 Materials

Polysorbate 20 high purity (PS20 HP) was obtained from Croda GmbH (Nettetal, Germany). Polysorbate hydrolases were produced in-house at Boehringer Ingelheim Pharma GmbH & Co. KG (Biberach an der Riss, Germany). Histidine and sodium chloride of parenteral grade were purchased from Sigma-Aldrich (St. Louis, USA).

7.2.2 Recombinant expression and purification of hydrolases

Recombinant hydrolases (Table 7.1) were recombinantly expressed and purified as previously described [131]. Each protein was produced using a stably expressing Chinese hamster ovary CHO cell line, followed by orthogonal affinity chromatography and polishing steps.

Table 7.1: Expressed and purified polysorbate hydrolases with abbreviations and their Uniprot accession numbers.

Uniprot Accession No.	Protein name	Gene name
A0A061I7X9	CES1F (Liver carboxylesterase B-1-like protein)	<i>Ces1f</i>
G3IIG1	CES2C (Carboxylic ester hydrolase 2C)	<i>Ces2c</i>
G3HKV9	LPLA2 (Group XV phospholipase A2)	<i>Pla2g15</i>
A0A8C2M2A7	PAF-AH (1-alkyl-2-acetylglycerophosphocholine esterase)	<i>Pla2g7</i>
G3HN89	PPT1 (Palmitoyl-proteinthioesterase 1)	<i>Ppt1</i>

7.2.3 Sample generation and degradation fingerprint acquisition

Each purified hydrolase was incubated individually in 10 mM histidine pH 6 containing $0.4 \text{ g} \cdot \text{L}^{-1}$ PS20 HP at room temperature in Eppendorf tubes (Eppendorf, Hamburg, Germany). To closely reflect actual conditions, a low hydrolase concentration of $20 \text{ ng} \cdot \text{mL}^{-1}$ was chosen (protein concentration determined by UV absorbance at 280 nm). To ensure broad kinetic coverage, up to 50 timepoints per hydrolase were collected. These timepoints were selected to capture enzymatic degradation from early to late stages. At each timepoint, samples were analyzed by RP-UPLC-MS and PS subspecies were extracted via generative modeling to quantify the relative abundance of different PS species [168]. Peak intensities were normalized to the initial timepoint (t_0) to generate degradation fingerprints for each hydrolase.

7.2.4 Fingerprint dataset preprocessing and feature selection

The complete dataset comprised normalized intensity values for up to 50 timepoints across 35 polysorbate (PS20) subspecies per hydrolase. During initial data evaluation, particularly in strongly degraded samples, outliers in PS subspecies were identified, suggesting potential inaccuracies in the extraction process. To ensure data reliability and analytical robustness, all subspecies were systematically re-assessed. Even subspecies with only very few outliers were removed to have a clean data input and not introduce biases for models evaluated. Only those subspecies that were consistently detectable, even in strongly degraded samples, were retained for further analysis. Since the hydrolytic degradation of PS always results in free Sorbitan, Isosorbide and POE species. Therefore, these subspecies are always present at high levels and do not contribute to differentiation between the hydrolases. Thus, these subspecies were not considered for classification.

This refinement reduced the number of subspecies from 35 to 23, a step taken to minimize bias and enhance the performance and interpretability of subsequent classification models. To focus the classification exclusively on degradation patterns, all sample profiles were normalized to the initial reference sample. This normalization strategy ensures that classification is based solely on the relative ratio among the 23 selected subspecies at each sampling timepoint. Consequently, metadata such as time, enzyme concentration, enzymatic activity, and overall PS concentration were excluded from the classification input, as they are not required for capturing the intrinsic degradation signature.

7.2.5 Model training and evaluation

All computational analysis were conducted in Python using scikit-learn, numpy, pandas and tensorflow. The processed fingerprint dataset was randomly divided into training (80 %) and test (20 %) subsets using stratified sampling to preserve class balance among the five hydrolases (CES1F, CES2C, LPLA2, PPT1, and PAF-AH).

Eight supervised classification models were evaluated for their ability to assign the correct hydrolase based on their individual degradation fingerprint. The models were tested for the following parameters listed in Table 2 and 3. The models and the best performing parameters are as followed: Logistic Regression (multi class: ovr, penalty: L2 regularization, solver: lbfgs), Random Forest (estimators: 120, bootstrap: False, max features: 3), Gradient Boosting (learning rate: 1.5, max depth = 3, 100 estimators), Support Vector Classifier (RBF kernel, C = 1.0). Additionally, three Artificial Neural Networks were implemented. More detailed specifications of the models are shown in Table 7.3. Hyperparameter tuning was performed using five-fold cross-validation on the training set. Performance metrics included accuracy, macro-averaged F1-score, and class-wise precision and recall.

Feature importance scores were extracted from tree-based models, highlighting which polysorbate species contributed the most to hydrolase differentiation.

Table 7.2: Hyperparameters evaluated for supervised classification models during model training and optimization.

Model	Hyperparameter	Values
Logistic Regression (logReg)	solver	['saga', 'lbfgs']
	multi_class	['ovr', 'multinomial']
	penalty	['l2', 'l1']
	C	['logspace(0,4,10)', 'logspace(0,4,15)', 'logspace(0,4,20)']
Random Forest (RFC)	n_estimators	[60, 90, 120, 150, 200]
	max_features	[2, 3, 4, 5, 10]
	bootstrap	[True, False]
Ada Boost (AB)	n_estimators	[50, 100, 150, 200]
	learning_rate	[0.01, 0.1, 0.2, 0.5]
Gradient Boosting (GB)	n_estimators	[5, 20, 40, 100]
	max_depth	[3, 5, 10, 20]
	learning_rate	[0.01, 0.1, 0.3, 0.6, 1.0, 1.5, 2.0]
Support Vector Classifier (SVC)	C	[0.01, 0.1, 1]
	kernel	['rbf', 'sigmoid']
	degree	[2, 3, 4]

Table 7.3: Architecture and configuration details of Artificial Neural Networks (ANNs) for hydrolase classification.

Model	Hidden Layers	Dropout	Output Activation	Loss Function	Optimizer	Classes	Mutually Exclusive
ANN-1	23 → 16	None	Softmax	Sparse Categorical Cross-Entropy	Adam	5	Yes
ANN-2	23 → 64 → 32 → 16	30%, 30%, 20%	Softmax	Sparse Categorical Cross-Entropy	Adam	5	Yes
ANN-3	23 → 64 → 32 → 16	30%, 30%, 20%	Sigmoid	Sparse Categorical Cross-Entropy	Adam	5	No

7.2.6 External validation and application to unknown samples

To evaluate the performance of the trained classification models, two types of external samples were analyzed. For the first scenario, which was used for validation, a formulation containing polysorbate was spiked with a known CHO-derived hydrolase, PAF-AH. This enzyme was prepared independently from the material used to build the model to ensure that the validation set was completely unseen. The spiked formulation was incubated in 10 mM Histidine at pH 6 and samples were collected at four incubation timepoints.

Each sample was analyzed by RP-UPLC-MS to generate a degradation fingerprint, which was then submitted to the trained models. This approach allowed assessment of whether the models could correctly identify the responsible hydrolase from a single measurement.

In a separate experiment, a purified and formulated monoclonal antibody was subjected to RP-UPLC-MS analysis to generate a degradation fingerprint. The host cell protein composition and hydrolase content of this formulation were unknown. The fingerprint was submitted to the trained model, and the model was used to assign the most likely hydrolase class based on the learned degradation patterns.

7.3 Results and Discussion

7.3.1 Overview of polysorbate hydrolase activity

To assess the activity of CHO-derived hydrolases under formulation relevant conditions, enzymes were incubated in a 10 mM histidine formulation (pH 6) at $0.4 \text{ mg} \cdot \text{ml}^{-1}$ PS20 HP. To reflect realistic levels that may occur in drug product formulations [71], $20 \text{ ng} \cdot \text{ml}^{-1}$ of the respective hydrolases were added to the formulations. Degradation was monitored over several weeks at 25°C , and the residual PS20 content was quantified by RP-UPLC-MS (Figure 7.2). Among the tested enzymes, CES1F, CES2C, LPLA2, PAF-AH and PPT1 exhibited clear hydrolytic activity. CES1F and LPLA2 showed the most rapid degradation, while PAF-AH was the least active among the five enzymes. These findings confirm previous reports that these hydrolases are capable of degrading polysorbates [106,131]. Importantly, the observed activity occurred at very low enzyme concentrations, highlighting their potential relevance for product stability.

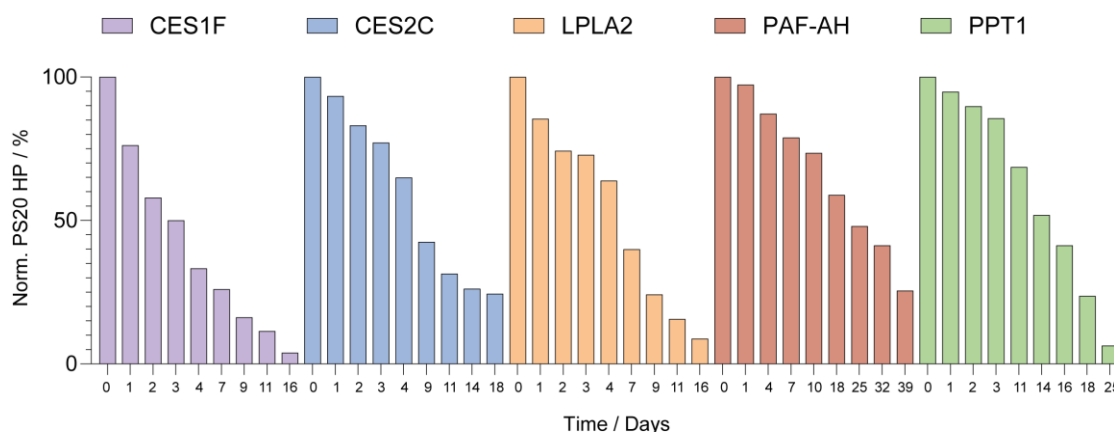


Figure 7.2: Degradation of PS20 HP ($0.4 \text{ mg} \cdot \text{mL}^{-1}$) by CHO derived hydrolases at $20 \text{ ng} \cdot \text{mL}^{-1}$. PS content was quantified via RP-UPLC-MS and normalized to the initial PS concentration. Each color represents a different hydrolase: CES1F (purple), CES2C (blue), LPLA2 (orange), PAF-AH (red) and PPT1 (green). Please note that only selected timepoints are shown for clarity.

It has been demonstrated that a number of hydrolases, including LPL, LIPA, CES1 and IAH1, are active against polysorbate [106,131]. These enzymes have also been tested here; however, at a concentration of $20 \text{ ng} \cdot \text{mL}^{-1}$, no measurable PS degradation was observed within the first weeks. Extended incubation of these samples led to oxidative degradation in the polysorbate blank. This indicates that their activity is lower compared to the five enzymes highlighted above. These enzymes were therefore not spiked at artificially high concentrations (in the $\mu\text{g} \cdot \text{mL}^{-1}$ range) and excluded from further fingerprinting and classification analysis. Nevertheless, the presented framework can be extended to incorporate additional enzymes as more data becomes available.

7.3.2 Distinct fingerprint patterns across hydrolases

To assess the separability of hydrolase-specific fingerprints, the normalized intensities of polysorbate species were visualized using pair plots. Each fingerprint corresponds to a single timepoint of a hydrolase incubation and represents normalized intensities of individual polysorbate species relative to the initial timepoint (t_0). Pair plots were generated using a subset of selected polysorbate species: S08, S12, I08, S12/12/14, S12/12/16, S10 and POE10 (Figure 7.2). These species were chosen based on their high feature importance scores (Figure 7.5) and chemical diversity, including both short- and long-chain esters. Each point represents the normalized intensities of two polysorbate species at one timepoint, resulting from the activity of one hydrolase. These visualizations allow the comparison of relative species abundances between enzymes. The diagonal histograms show the distribution of each species individually by hydrolase class. Notably, several species combinations exhibit well-separated clusters, indicating class-specific degradation patterns.

CES2C and CES1F form compact and for some of the species non-overlapping clusters. This suggests that, despite their shared classification as carboxylesterases, the two enzymes

generate distinct degradation patterns for some of the PS subspecies. Especially in plots like S12 vs I08, CES1F and CES2C show well-defined separable clusters. This supports previous biochemical findings that CES1F and CES2C differ in their substrate preferences [207].

PAF-AH forms a distinct cluster in the S08 vs S12 plot, indicating it can be differentiated from other enzymes using specific species combinations.

LPLA2 shows broader or even bimodal profiles for several species such as POE10 and S12. This may be due to non-linear degradation kinetics, where initially a rapid degradation (high affinity towards certain subspecies) can be observed which slows down over time (subspecies with little affinity are hydrolyzed). In some scatter plots, the data points, which represent the paired normalized intensities of two polysorbate species, are spread across the full range, showing high variability but still with a recognizable pattern.

PPT1 shows a preference for triester species, especially S12/12/14 and S12/12/16. This is reflected in the diagonal histograms, where the intensities are close to zero, indicating rapid and consistent degradation of these species. In contrast, monoesters such as S10 and S12 retain high intensities, suggesting low activity on these substrates. In scatter plots that include triesters, PPT1 forms a distinct cluster, making it clearly distinguishable from the other hydrolases due to its pronounced triester selectivity.

Overall, this visualization illustrates the differences between hydrolase degradation fingerprints. As the data is normalized to the initial timepoint, the observed differences are independent of the absolute degradation time or enzyme concentration, allowing direct comparison between fingerprints even when the timepoint is unknown. The class-specific clustering across multiple species suggests that polysorbate degradation fingerprints contain sufficient discriminatory information for hydrolase classification and provide the basis for supervised model training.

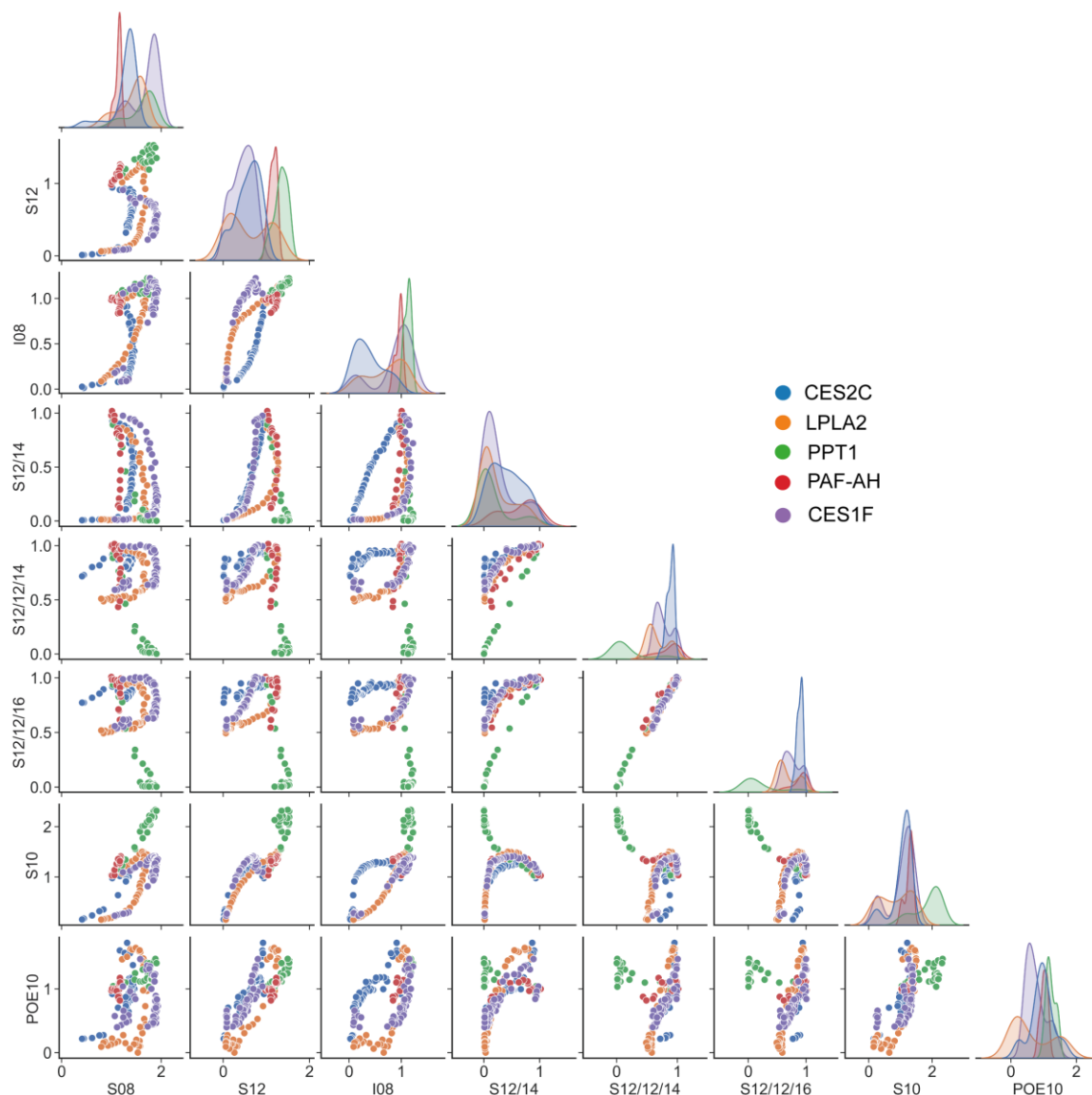


Figure 7.3: Pair plots of selected polysorbate species. Each point represents a single fingerprint for one timepoint and one hydrolase. The selected species were chosen based on their contribution to classification performance (feature importance) and chemical representativeness. Colors indicate responsible hydrolase. Axes show normalized intensities.

7.3.3 Classification model performance

To evaluate whether the degradation fingerprints are sufficient to predict the responsible hydrolase, different classification approaches were compared (Table 7.2 and Table 7.3). Models were trained on normalized fingerprints from five CHO-derived hydrolases, using 80 % of the dataset for training and 20 % for testing. Each fingerprint represented a single timepoint and contained normalized intensities of 23 polysorbate species. Due to the normalization only the ratios at a specific timepoint of the used PS20 species is evaluated, therefore the classification is independent of the PS concentration itself. Furthermore, no

information on incubation time or enzyme concentration was included, ensuring that predictions relied solely on the degradation pattern.

The models evaluated in this study were Logistic Regression (logReg), Random Forest Classifier (RFC), Gradient Boosting (GB), Ada Boost (AB), Support Vector Classifier (SVC), and three Artificial Neural Networks (ANNs) of either increasing depth or designed for multi-class classification (Table 7.2 and Table 7.3).

Figure 7.4 shows the confusion matrices for the results of the seven top-performing models and for Ada Boost. Logistic Regression, Random Forest, Gradient Boosting, Support Vector Classifier and all three ANNs achieved perfect classification (accuracy = 1), producing identical confusion matrices. These results confirmed that even closely related fingerprint patterns (e.g. CES1F and CES2C) could be reliably separated without misclassifications. Ada Boost showed slightly lower performance (accuracy = 0.92), with occasional misclassifications between CES1F and CES2C and one misclassification of LPLA2 as CES1F. Although pair plots of selected species (Figure 7.3) reveal distinct clusters for CES1F and LPLA2, this model still misclassified these enzymes.

Overall, the results demonstrate that all evaluated models, except Ada Boost, can accurately identify the enzyme from a single timepoint fingerprint, confirming that the relevant discriminatory features are embedded in the degradation pattern itself rather than requiring kinetic information.

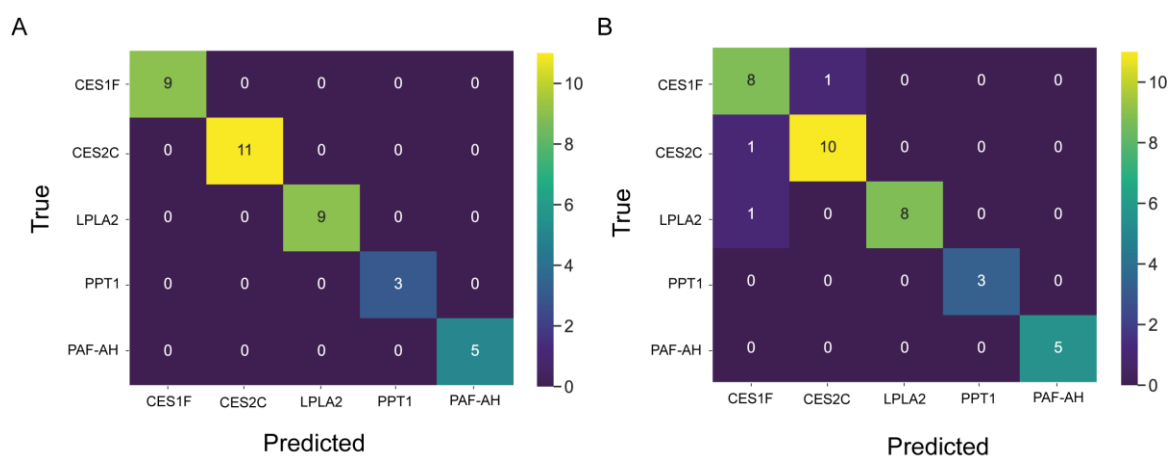


Figure 7.4: Confusion matrices for classification models trained on single-timepoint degradation fingerprints of five CHO-derived polysorbate hydrolases. A: Logistic Regression, Random Forest, Gradient Boosting, Support Vector Classifier, and three Artificial Neural Networks (ANN-1 to ANN-3). B: Ada Boost. All models were evaluated on an internal test set. Confusion matrices display class-wise prediction results for all five hydrolases: CES1F, CES2C, LPLA2, PAF-AH and PPT1.

7.3.4 Feature importance and contribution of PS species

To understand which species contributed most to classification performance, feature importance scores were extracted from the trained Random Forest model (Figure 7.5). These

scores reflect how often and how effectively each species was used to split the data during training. A higher importance score indicates that the species was frequently used in decision splits and significantly improved class separation. Among these, the monoester S08 emerged as the most informative feature, followed by the triester species S12/12/14 and S12/12/16, and the isosorbide-based species I08. Additional contributors included S12 and S12/18, although their influence was comparatively lower.

This matches previous observations in the pair plots, where enzymes showed specific patterns in these species. For example, PPT1 consistently led to a rapid and complete depletion of triesters, resulting in near-zero intensities across timepoints. This makes these species particularly useful for distinguishing between hydrolases. PAF-AH showed a pronounced degradation of the monoester S08 while CES2C and CES1F showed distinct patterns for combinations with I08 species.

From a practical perspective, the feature importance patterns suggest that only a subset of polysorbate species is required for robust enzyme classification. This indicates that future models could be built on a reduced set of species, potentially decreasing data complexity while maintaining classification accuracy.

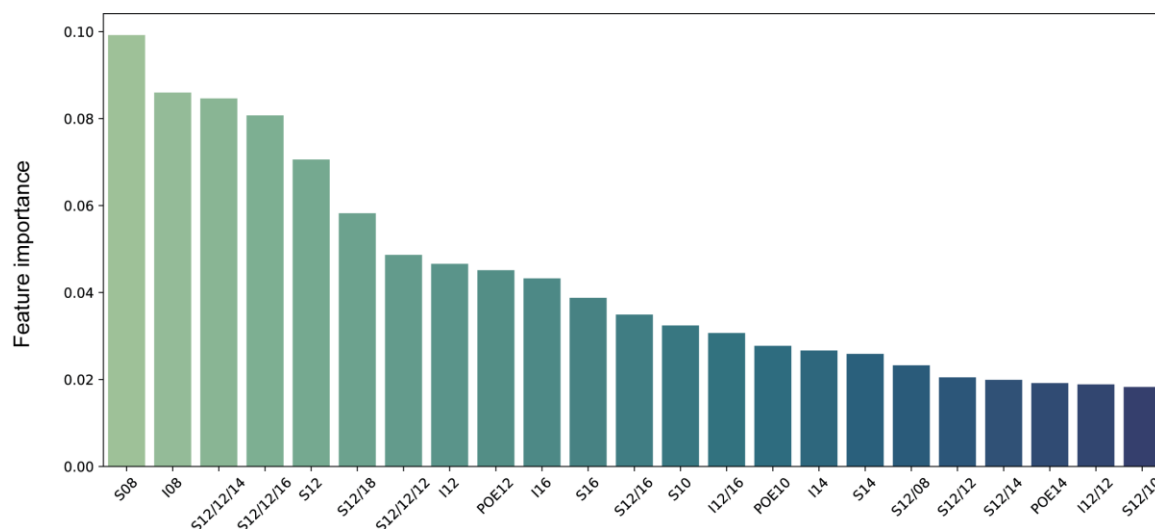


Figure 7.5: Feature importance scores of individual polysorbate species derived from the Random Forest model. Bar plots display the relative importance of selected polysorbate species as determined by the trained random forest classifier. Feature importance reflects how frequently and effective each species was used to split decision trees during model training. Higher values indicate stronger contribution to hydrolase classification.

7.3.5 External validation with spiked samples

To assess the robustness of the classification models beyond the internal test set, an external validation was performed using a formulation sample spiked with the hydrolase PAF-AH. This sample was generated independently of the training dataset and was analyzed at four individual timepoints (t1-t4). The goal was to assess whether the trained models could correctly assign the enzyme class based on the Polysorbate 20 fingerprint. Table 7.3

summarizes the classification probabilities for each model across the four timepoints. The results demonstrated that most models correctly identified PAF-AH at early degradation stages with high confidence, while prediction certainty decreases at later timepoints (Figure 7.6).

This observation indicates that fingerprint-based classification has inherent limitations at advanced degradation stages. Initially, degradation patterns are enzyme-specific and well-defined, allowing models to confidently assign classes. However, as degradation progresses, many species are already degraded, and the fingerprints become less distinctive. Consequently, specific fingerprint markers disappear and become non-detectable, reducing classification accuracy and increasing the likelihood of misclassifications. This highlights the importance of considering degradation stage when applying fingerprint-based approaches.

Gradient Boosting and Logistic Regression achieved perfect or near-perfect confidence for the first two samples, whereas confidence dropped substantially for the last sample, which was misclassified by Gradient Boosting as PPT1 with full certainty. Random Forest and Support Vector Classifier also showed declining confidence over time, although Support Vector Classifier maintained correct predictions across all four samples. Ada

Boost exhibited moderate performance, correctly classifying early samples but misclassifying the latest timepoint.

ANN-2 and ANN-3 maintained high confidence for PAF AH across all timepoints, outperforming several tree-based models, while ANN-1 showed inconsistent predictions and even misclassified late-stage samples. A likely reason is that ANN-1 has only one hidden layer and is therefore too shallow to capture the more complex patterns in the data. ANN-2 and ANN-3, which each contain three hidden layers, seem to extract these features more effectively. This allows them to correctly classify PAF-AH even when PS20 is already heavily degraded.

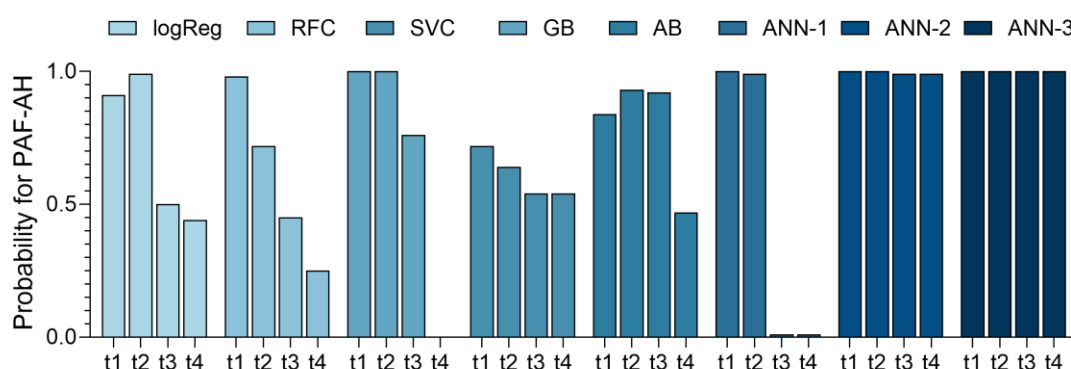


Figure 7.6: Predicted probability for an external PAF-AH spike sample. Predictions are shown for four samples taken at increasing degradation timepoints (sample t1 = earliest, sample t4 = latest) for eight models: Logistic Regression (logReg), Random Forest (RFC), Gradient Boosting (GB), Support Vector Classifier (SVC), Ada Boost (AB), and three

Artificial Neural Networks (ANN-1, ANN-2, ANN-3). Bars are color coded by model. Most models show high confidence at early timepoints, while confidence decreases at later stages, reflecting reduced fingerprint specificity due to extensive degradation.

These findings have important implications for more complex scenarios, such as mixtures of hydrolases in formulated drug products. At advanced degradation stages, fingerprints may reflect overlapping activity patterns from multiple enzymes, reducing the distinctiveness of any single class. Models that rely predominantly on a small set of dominant features, such as tree-based classifiers, tend to perform poorly under these conditions. In contrast, deeper neural networks may benefit from their ability to integrate information across a large number of features, enabling them to better approximate mixed signal patterns. To address this challenge, ANN-3 was trained as a multilabel classifier, allowing it to predict multiple independent labels (hydrolases) for a single input instance and to treat each label as an independent binary decision.

As shown in Table 7.4, ANN-3 also assigns high likelihood values to PPT1, especially at later sampling timepoints. This occurs because both hydrolases have stronger preferences for di- and triesters compared to the other investigated hydrolases. A more general, but also more complex solution would be to incorporate training data that mimic mixed-enzyme conditions into the current framework to tackle the problem when having more than one hydrolase in the same sample. This could be achieved for example by generating synthetic fingerprints that combine profiles from different hydrolases. Such an approach would help improve classification robustness when multiple enzymes contribute simultaneously to polysorbate degradation.

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Table 7.4: Model-based classification probabilities for external test data using a PAF-AH spiked sample.

Model	Sample	CES1F	CES2C	LPLA2	PPT1	PAF-AH
Logistic Regression	t1	0.00	0.00	0.00	0.00	0.91
	t2	0.00	0.00	0.00	0.01	0.99
	t3	0.00	0.00	0.00	0.50	0.50
	t4	0.00	0.00	0.00	0.56	0.44
Random Forest						
	t1	0.01	0.00	0.01	0.00	0.98
	t2	0.00	0.00	0.08	0.20	0.72
	t3	0.01	0.06	0.06	0.47	0.45
	t4	0.02	0.21	0.21	0.48	0.25
Gradient Boosting						
	t1	0.00	0.00	0.00	0.00	1.00
	t2	0.00	0.00	0.00	0.00	1.00
	t3	0.00	0.00	0.00	0.24	0.76
	t4	0.00	0.00	0.00	1.0	0.00
Support Vector Classifier						
	t1	0.03	0.11	0.08	0.07	0.72
	t2	0.03	0.07	0.05	0.22	0.64
	t3	0.04	0.06	0.05	0.31	0.54
	t4	0.04	0.07	0.07	0.28	0.54
Ada Boost						

t1	0.00	0.16	0.00	0.00	0.84
t2	0.00	0.00	0.00	0.07	0.93
t3	0.00	0.00	0.00	0.08	0.92
t4	0.00	0.01	0.00	0.52	0.47
ANN-1					
t1	0.00	0.00	0.00	0.00	1.00
t2	0.00	0.00	0.00	0.01	0.99
t3	0.00	0.00	0.00	0.99	0.01
t4	0.00	0.00	0.00	0.99	0.01
ANN-2					
t1	0.00	0.00	0.00	0.00	1.00
t2	0.00	0.00	0.00	0.00	1.00
t3	0.00	0.00	0.00	0.01	0.99
t4	0.00	0.00	0.00	0.01	0.99
ANN-3					
t1	0.00	0.49	0.01	0.56	1.00
t2	0.00	0.13	0.01	0.83	1.00
t3	0.00	0.01	0.00	1.00	1.00
t4	0.00	0.02	0.13	0.95	1.00

7.3.6 Prediction of unknown samples

To evaluate the applicability of the classification models beyond controlled conditions, we applied them to degradation fingerprints derived from a monoclonal antibody formulation. These model antibodies were purified under a typical downstream purification process and contained polysorbate 20 HP as part of the formulation. The composition and abundance of polysorbate hydrolases in these samples are unknown, making them a realistic test case for fingerprint-based classification. Degradation fingerprints were generated at four

incubation timepoints (2 months and 4 months at 25 °C, and 15 months at 5 °C and 25 °C). Following data extraction and normalization, the fingerprints were submitted to the trained classification models.

The classification results are shown in Figure 7.7. CES2C had the highest mean probability at all timepoints (0.63-0.92), indicating that it is the most likely contributor to polysorbate degradation in the tested mAb formulation. The second highest probability was observed for CES1F.

At the first timepoint (two months), CES2C was not clearly dominant, which may indicate that degradation was still in the early stages and that the fingerprint was not yet specific enough. This is supported by the fact that CES1F and CES2C are both carboxylesterases with a preference for monoester species, making them difficult to distinguish when only minor changes have occurred. At the 15-month sampling timepoint at 25 °C storage condition, the confidence for CES2C decreased again. This is expected when a subset of polysorbate subspecies has already been fully hydrolyzed, because fewer informative species remain and the fingerprint provides less differentiation between enzymes, making the signal less pronounced. In general, classification is most challenging when degradation is either minimal or advanced, because many values are close to one or zero, which reduces class separability. For samples with very little change, only a few of the 23 polysorbate species differ from the reference. In this case, random forest trees may split on features that remain almost unchanged, which can lead to less reliable predictions. If only six of the 23 species show any deviation, the model may select features with values still close to 1, yielding near-random assignments for individual trees. Aggregation across 100 estimators stabilizes the overall prediction, but the confidence remains lower. This makes the random forest less reliable at these stages compared to other applied algorithms here. Based on the probabilities for LPLA2, PPT1 and PAF-AH (Figure 7.7), these enzymes are unlikely to play a critical role in this sample. Overall, the results suggest that CES2C is the most likely contributor to polysorbate degradation in this sample, with CES1F possibly contributing as well. A key advantage of the models is their ability to clearly exclude hydrolases that are not relevant for polysorbate degradation based on their mismatch with the observed fingerprints.

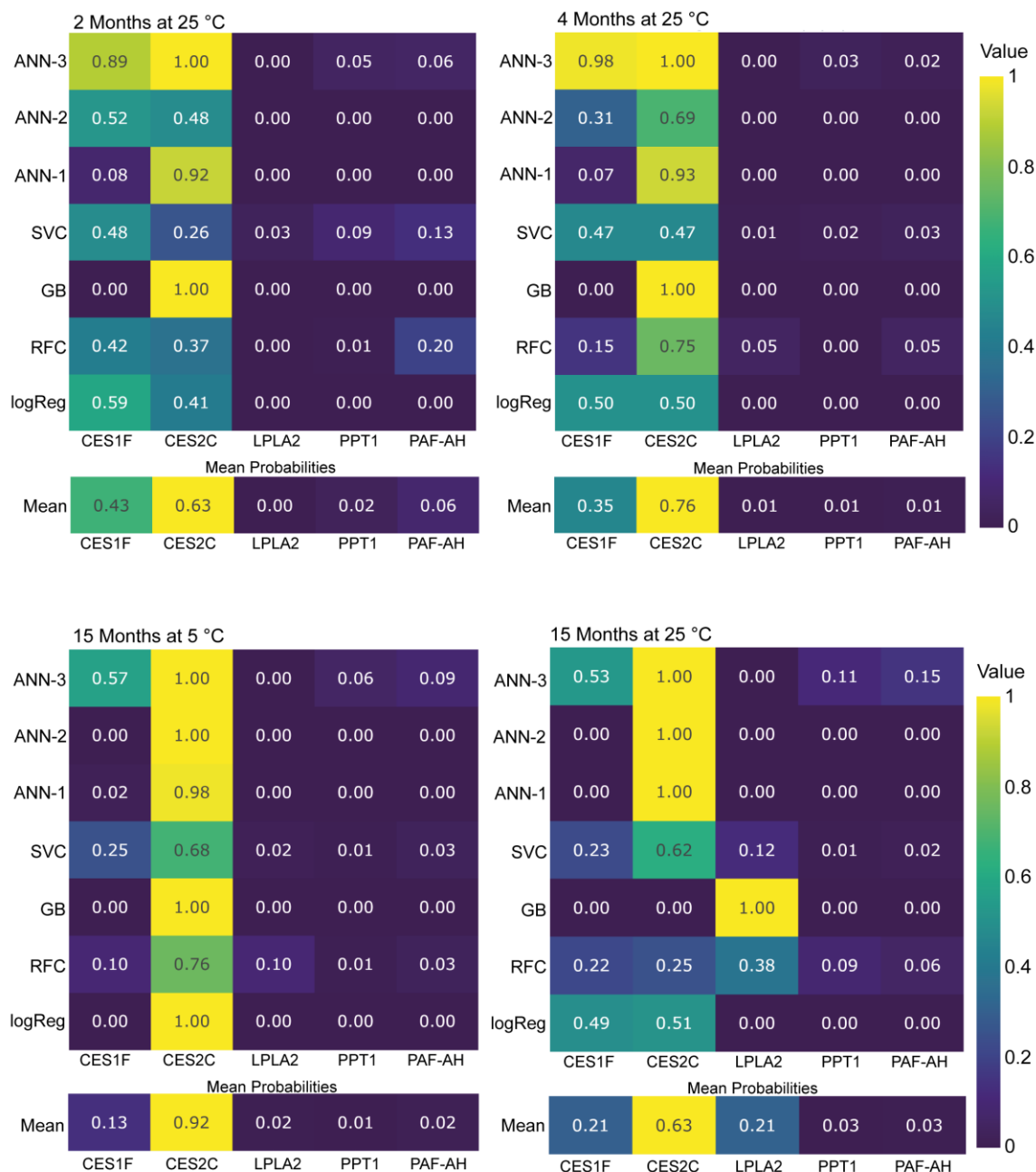


Figure 7.7: Model-based classification probabilities for a monoclonal antibody formulation at four sampling points. Panels show heatmaps of predicted class probabilities for five CHO-derived polysorbate hydrolases (CES1F, CES2C, LPLA2, PPT1, PAF-AH) generated by seven classifiers at 2 months (25 °C), 4 months (25 °C), 15 months (5 °C) and 15 months (25 °C). Each cell represents the probability assigned by one model for one hydrolase at the given timepoint. The bottom row in each panel displays the mean probability across all models. Colors encode probability from low (dark) to high (yellow).

The fingerprint-based classification approach presented in this study offers several applications to improve biopharmaceutical development. Fingerprint data enables the identification of hydrolases that consistently contribute to polysorbate degradation across formulations. If certain enzymes emerge as dominant contributors while others show negligible

activity, this supports risk-based prioritization of hydrolases. These findings can directly guide downstream process development. For example, polishing steps such as ion exchange or hydrophobic interaction chromatography can be systematically optimized to selectively remove high-risk hydrolases [130]. Furthermore, the method allows comparison of different purification trains [112]. By analyzing degradation fingerprints from the same monoclonal antibody purified through different downstream processes, it can be systematically assessed whether certain hydrolases are consistently removed or retained. This allows the evaluation of which purification strategy is most effective at mitigating specific enzymes.

The approach also supports cell line engineering. Once high-risk hydrolases are identified, targeted gene knockouts can be implemented to eliminate their expression in CHO production cell lines. This strategy has already been applied successfully to reduce polysorbate degradation and improve product stability [32,217,230].

Beyond process optimization, fingerprint analysis provides insights into hitchhiking phenomena where specific hydrolases co-purify with certain mAbs due to molecular interactions [75]. If a hydrolase consistently appears in fingerprints across multiple purification trains for a given antibody, this may be indicative of a hydrolase-antibody binding interaction. Conversely, if the same purification strategy yields different fingerprint profiles for different antibodies, this suggests molecule-specific effects that cannot be addressed by standardized purification approaches.

Recent studies indicate that certain host cell-derived hydrolases can also associate with protein aggregates in formulated drug products [77,241]. This observation highlights a critical limitation of conventional HCP monitoring approaches, which measure protein abundance but do not provide information on functional activity. Enzymes embedded in aggregates may be detected by proteomics, yet their actual contribution to polysorbate degradation remains uncertain. Conversely, low abundance hydrolases with high enzymatic activity may not only remain undetected but are often underestimated.

Importantly, fingerprint-based classification can be combined with LC-MS-based HCP profiling to strengthen enzyme identification. While proteomics provides broad coverage, it may miss low-abundance enzymes that are functionally active. This can be due to the extreme dynamic range in monoclonal antibody drug products [68,226]. Integrating both methods could provide a more complete picture of degradation risk and support systematic mitigation strategies.

A current limitation of the framework is that models were trained exclusively on single-enzyme profiles. In real formulation samples, however, multiple hydrolases may be present simultaneously, either in additive or overlapping activity. This is particularly relevant at late degradation stages, where multiple hydrolases may have contributed to the degradation and where model confidence tends to decline. In such cases, the fingerprint may not match one enzyme perfectly but instead represent a hybrid pattern, resulting in lower confidence scores or different class assignments at different timepoints within the sample. While the framework performs well for isolated enzymes, its interpretability decreases in the presence of complex mixtures. In these scenarios, ANN-3 (multi-label classifier) is

better suited than strictly exclusive models because it does not force a single class and can indicate partially overlapping patterns. However, since it was trained only in single-enzyme profiles, such indications should not be interpreted as definitive evidence of mixtures. Moreover, when high-confidence assignment is not possible, predictions can still narrow down the set of likely candidates. For example, if the model consistently excludes certain enzymes across multiple timepoints. This enables a targeted focus on a smaller subset of hydrolases for further investigation.

One possible extension of the current approach would be to train the model on simulated mixtures of enzyme fingerprints. For instance, by linearly combining normalized degradation profiles from two or more hydrolases. This could help the model to learn intermediate patterns and help improve classification robustness in mixed samples.

In future work, predictions on unknown samples may be complemented by orthogonal methods such as LC-MS-based HCP profiling, allowing experimental confirmation of predicted enzyme identities and refinement of classification in complex mixtures.

7.4 Conclusion and Outlook

This work establishes degradation fingerprinting as a robust and functional approach for classifying polysorbate-degrading hydrolases. The approach enables enzyme identification from single RP-UPLC-MS measurements, independent of enzyme concentration or degradation time, demonstrating that the degradation pattern itself contains sufficient discriminatory information.

The current study focused on five highly active CHO hydrolases which represent the main contributors to polysorbate hydrolysis at formulation relevant conditions. Additional hydrolases with lower activity were excluded but can readily be integrated into the classification framework as more data become available.

Future work should extend the current framework to mixed-enzyme samples, as multiple hydrolases may act simultaneously in real formulations. Generating in-silico mixed fingerprints could improve model robustness for overlapping degradation profiles. Combining fingerprint-based classification with orthogonal analytical methods such as HCP proteomics may further strengthen enzyme identification in complex samples.

Overall, the method presented here offers a valuable tool for risk-based prioritization of polysorbate degrading hydrolases. Fingerprint-based classification can help identify enzymes that are functionally active under formulation conditions and thus guide targeted mitigation of polysorbate degradation in therapeutic formulations.

Acknowledgements

We would like to thank Holger Thie for excellent project management and support throughout the study.

8 Conclusion and Outlook

This dissertation consolidates multiple perspectives to address one of the most persistent challenges in biopharmaceutical development: polysorbate degradation caused by host cell hydrolases. It integrates complementary analytical and mechanistic approaches to build a comprehensive understanding of polysorbate-degrading host cell hydrolases. The following sections summarize the key findings, highlight current limitations, and outline future research directions that emerge from this work.

8.1 Polysorbate-Degrading Host Cell Hydrolases in Biopharmaceuticals

Enzymatic polysorbate degradation is driven by multiple host cell hydrolases rather than a single enzyme. Across five studies, this work integrates insights into enzyme identity, downstream behavior, interaction properties, and functional activity, demonstrating how these elements collectively explain why certain hydrolases persist throughout biopharmaceutical manufacturing and how they ultimately contribute to product instability.

Chapter three laid the foundation by identifying a discrete set of polysorbate-degrading hydrolases that were consistently detected in monoclonal antibody formulations. These findings challenge earlier assumptions that degradation is driven by a single enzyme and instead show that several hydrolases can contribute to polysorbate degradation. This narrowed the focus to a defined group of enzymes and enabled a more systematic and targeted investigation of polysorbate-degrading hydrolases.

Chapters four and five extend this understanding into downstream processing. Chapter four showed that hydrolase persistence is strongly influenced by physicochemical properties that lead to co-elution during polishing. This explains why certain hydrolases repeatedly appear in final drug substances across different molecules and why they are difficult to remove using standard purification strategies. Chapter five introduced an orthogonal mechanism by demonstrating that some hydrolases can interact reversibly with antibodies. These interactions provide a mechanistic explanation for hitchhiking during Protein A capture and address an important gap that co-elution alone cannot explain. When viewed together, the results from chapters four and five show that hydrolase persistence results from multiple mechanisms acting across different purification stages. Hitchhiking during capture enables some hydrolases to enter the downstream process, while co-elution during polishing determines how long they remain. Importantly, hydrolases without detectable

affinity may still survive the capture step through additional pathways such as weak resin interactions or aggregate association. This cumulative interplay of hitchhiking and co-elution challenges conventional purification strategies, as clearance cannot be predicted by resin selectivity alone. Consequently, effective mitigation requires a holistic view of the entire purification train, considering how early capture behavior influences subsequent separation during the polishing steps. These insights underscore the need for tailored strategies that combine process optimization approaches to disrupt both hitchhiking and co-elution phenomena.

Chapter six and seven extend the perspective from persistence to functional activity. Both chapters focus on hydrolases identified in Chapter three as active against polysorbates. Chapter six demonstrated that polysorbate degradation by CHO-derived hydrolases results in distinct enzymatic fingerprints, which can be resolved at the subspecies level using RP-UPLC-MS. These fingerprints revealed clear differences in substrate preferences such as mono-, di-, and multiester, and highlight why indirect assays often misinterpret enzymatic activity.

Chapter seven builds on this by introducing a machine-learning framework that provides a promising approach for classifying hydrolases based on their degradation fingerprints. By training supervised models on normalized degradation profiles, the study demonstrates that hydrolase identity can be predicted from a single measurement, independent of enzyme concentration or degradation time. This represents a significant advance over traditional abundance-based proteomics, which do not allow differentiation between active enzymes from inactive or aggregated forms. The ability to classify hydrolases based on functional fingerprints rather than their presence alone offers a practical tool for risk-based prioritization.

Although this dissertation defines a recurring group of polysorbate hydrolases, additional enzymes may still contribute to degradation. The frameworks established here should therefore be viewed as iterative and extendable. As analytical sensitivity improves and new hydrolases are discovered, these can be integrated into the existing framework.

While this work provides comprehensive mechanistic insights, several aspects reflect current analytical constraints and warrant further investigation. Downstream characterization studies presented here were performed using recombinantly expressed hydrolases. This approach was necessary because endogenous hydrolases could not be reliably quantified at the concentrations typically present in late-stage process intermediates. Current mass-spectrometry based methods often lack the required sensitivity under these conditions, particularly in the presence of high antibody concentrations, and generally do not achieve detection limits below 0.1 ppm [225]. Chapter six demonstrated that hydrolases can remain highly active even at concentrations well below current analytical detection limits. This highlights the critical need for more sensitive quantification methods. Furthermore, commercial ELISAs targeting specific CHO-derived polysorbate hydrolases are not available, which further limits the ability to monitor endogenous enzymes during purification.

Once robust and specific quantification methods become available, future studies can complement the findings presented here by refining the characterization of endogenous hydrolases during polishing and across additional unit operations. Such extensions would not only deepen mechanistic understanding but also enable predictive control strategies throughout the entire bioprocess.

8.2 Future Research Perspectives

The insights gained in this dissertation open several promising directions for future research. One area that is likely to benefit most from the identification of a defined set of polysorbate-degrading hydrolases is cell line engineering. While single-gene knockouts have already proven effective for reducing specific hydrolase activities, further strategies will likely focus on multiplex knockouts targeting several enzymes simultaneously. However, such approaches must be implemented with particular care: any engineered host cell line must retain comparable growth behavior, productivity, and product quality attributes, including glycosylation profiles. It is equally important to ensure that genetic modifications do not trigger compensatory upregulation of other unwanted proteins, which could introduce new risks for product stability or immunogenicity. Comprehensive characterization of engineered cell lines will therefore be essential.

On the downstream side, the integration of fingerprint-based enzyme identification with targeted quantification approaches represents a particularly promising direction. The polysorbate degradation fingerprints described in this work provide a functional readout that directly indicates which enzyme is catalytically responsible, even at concentrations far below the sensitivity of current mass-spectrometry-based HCP methods. This represents a major advantage over abundance-based detection, which cannot distinguish enzymes that are present but inactive from those that truly drive degradation. Future research should therefore focus on developing hydrolase-specific quantification assays capable of detecting endogenous CHO enzymes directly in process intermediates. When combined with functional fingerprints, such assays would allow not only concentration measurements but also assessment of the actual catalytic relevance of each enzyme. This would reduce the risk of monitoring the “wrong” hydrolase and provide a far more accurate basis for risk-based prioritization during process development.

A further direction for future work could involve integrating hydrolase-specific information into mechanistic chromatography models for downstream process development. Mechanistic modeling enables the simulation of chromatographic operations as a function of varying process parameters. This enables a predictive understanding of how changes in operating conditions affect product quality attributes. As incorporating the entire HCP pool is challenging due to the large number of proteins with diverse behaviors, focusing on specific critical proteins offers a more feasible path [42]. If hydrolase-specific quantification assays become available, these enzymes could be incorporated as explicit species within such models. This may enable the prediction of hydrolase co-elution behavior under different polishing conditions, rather than relying on extensive experimental screening. It may help

identify optimal operating windows and would represent a valuable step toward more predictive downstream process development.

Finally, the hitchhiking assay developed in this dissertation provides a foundation for exploring molecular strategies to reduce HCP-mAb interactions, potentially extending into antibody engineering [161]. Understanding which motifs promote transient binding could guide the design of antibodies or antibody variants with reduced HCP association, offering an additional avenue for impurity control at the molecular level.

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Abbreviations

AB	AdaBoost Classifier
ABPP	Activity-Based Protein Profiling
AC	Affinity chromatography
ACN	Acetonitrile
AEX	Anion-exchange chromatography
ANN	Artificial Neural Network
AHC	Anti-Human Constant biosensor (trade name)
ANOVA	Analysis of variance
API	Active Pharmaceutical Ingredient
BLI	Biolayer Interferometry
BSA	Bovine Serum Albumin
CAD	Charged Aerosol Detection
CDR	Complementarity-determining region
CES1	Liver carboxylesterase 1-like protein
CES1(F)	Carboxylesterase 1 (F)
CES2C	Carboxylic ester hydrolase 2C
CEX	Cation-exchange chromatography
CH1-3	Heavy chain constant domain 1-3
CHO	Chinese Hamster Ovary
CMC	Chemistry, Manufacturing, and Controls
CMC (surfactant)	Critical Micelle Concentration
CPP	Critical process parameter

CQA	Critical quality attribute
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9
CV	Column volume
DDA	Data-Dependent Acquisition
DIA	Data-Independent Acquisition
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DoE	Design of experiments
DSP	Downstream processing
DTT	Dithiothreitol
EC	Enzyme Commission
ELISA	Enzyme-linked immunosorbent assay
ELSD	Evaporative Light Scattering Detector
EP	European Pharmacopeia
ESI	Electrospray Ionization
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FMA	Fluorescence micelle assay
FT	Flow-through
GB	Gradient Boosting
gDNA	Genomic DNA
Gns	N-acetylglucosamine-6-sulfatase
GS	glutamine synthetase
HC	Heavy chain
HCP	Host cell protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIC	Hydrophobic interaction chromatography
His	Histidine
His-tag	Polyhistidine-tag
HMW	High molecular weight
HP	High Purity
HRP	Horseradish Peroxidase
HTS	High-throughput screening
Iah1	Isoamyl acetate-hydrolyzing esterase 1-like
IEX	Ion-exchange chromatography
IgG	Immunoglobulin G
IMAC	Immobilized Metal Ion Affinity Column
KD	Dissociation Constant
KO	Knockout
Kp	Partition Coefficient
LC	Light chain
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LIPA	Lysosomal acid lipase
LOD	Limit of detection
LOQ	Limit of quantification
logReg	Logistic Regression
LPL	Lipoprotein lipase
LPLA2	Group XV phospholipase A2
mAb	Monoclonal antibody
MMC	Mixed-mode chromatography
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
MST	Microscale thermophoresis

MUD4	4-methylumbelliferyl decanoate
MW	Molecular Weight
NPN	N-Phenyl-1-naphthylamine
NSB	Non-specific binding
PAF-AH	Platelet-Activating Factor Acetylhydrolase
PDB	Protein Data Bank
pI	Isoelectric point
PCR	Polymerase Chain Reaction
PLD3	Phospholipase D3
PLBL2	Phospholipase B-Like 2
POE	Polyoxyethylene
ppm	Parts per million
PPT1	Palmitoyl-proteinthioesterase 1
ProtA	Protein A
PS	Polysorbate
PS20 / PS80	Polysorbate 20 / 80
QDa	Single Quadrupole Mass Detector (trade name)
QSPR	Quantitative structure-property relationship
RFC	Random Forest Classifier
RP-LC	Reversed-phase liquid chromatography
SAX	Streptavidin High Precision biosensor (trade name)
scFv	Single-chain fragment variable
SD	Standard deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC	Size exclusion chromatography
SMPD1	Sphingomyelin phosphodiesterase
SPR	Surface plasmon resonance

SRM	Selected Reaction Monitoring
Strep-tag	Strep-Tactin affinity tag
SVC	Support Vector Classifier
TIC	Total Ion Chromatogram
TPM	Transcripts per Million
Tris	Tris(hydroxymethyl)aminomethane
UF/DF	Ultrafiltration / Diafiltration
USP	Upstream processing
USP (pharmaco- peia)	United States Pharmacopeia
UV	Ultraviolet
VCD	Viable Cell Density
VH	Heavy chain variable domain
VL	Light chain variable domain
WP	Weak partitioning

Appendix A Supplementary Material Chapter 3

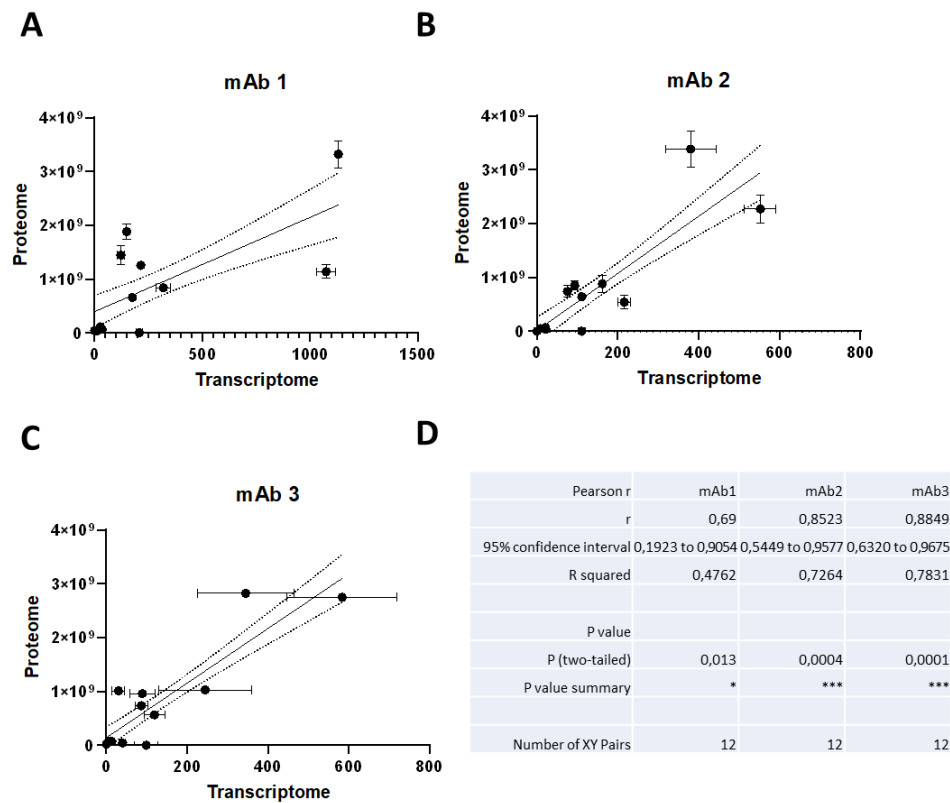


Figure A1: Correlation analyses of transcript and protein expression data of investigated hydrolases for monoclonal antibody (mAb)1 (A), mAb2 (B) and mAb3 (C) producer cell line. (D) Numerical values for correlation analyses.

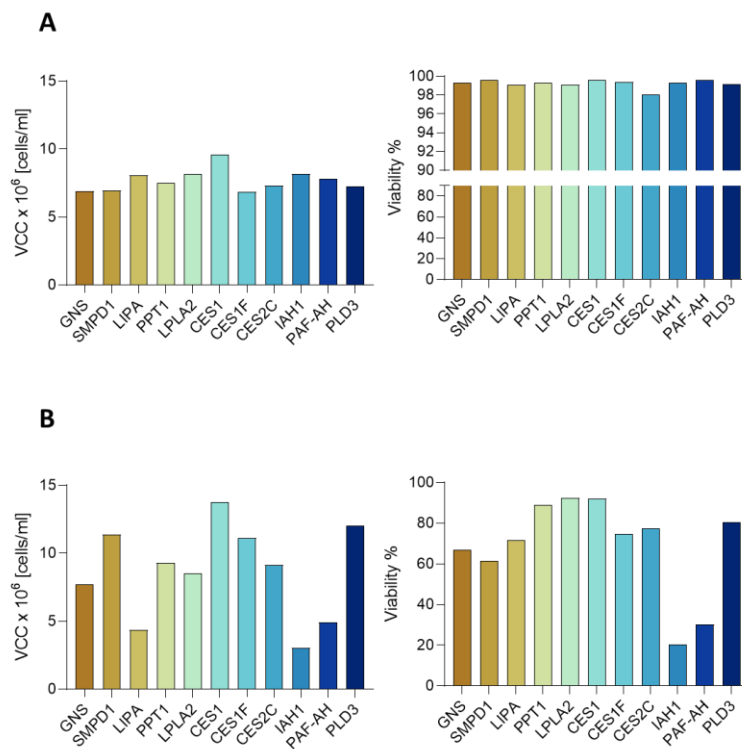


Figure A2: A: Viable cell concentration (VCC) and viabilities of hydrolase expressing CHO K1 LPL knockout (KO) pools at time of batch harvest for secretion analysis. A: VCC and viabilities of hydrolase expressing CHO K1 LPL KO pools at time of (fed-) batch harvest for hydrolase purification.

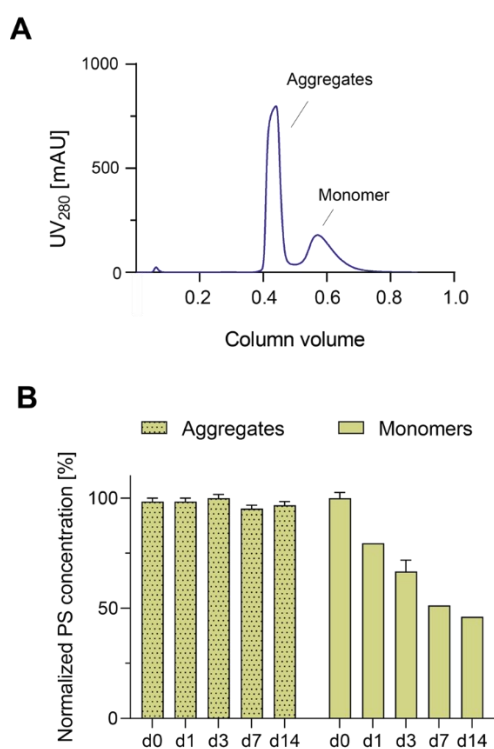


Figure A3: Instability and aggregation propensity of PPT1. A: Size exclusion chromatogram of PPT1, separating the aggregated species from monomeric PPT1. B: Polysorbate degradation over time for the monomer pool and the aggregate pool derived from the size exclusion run.

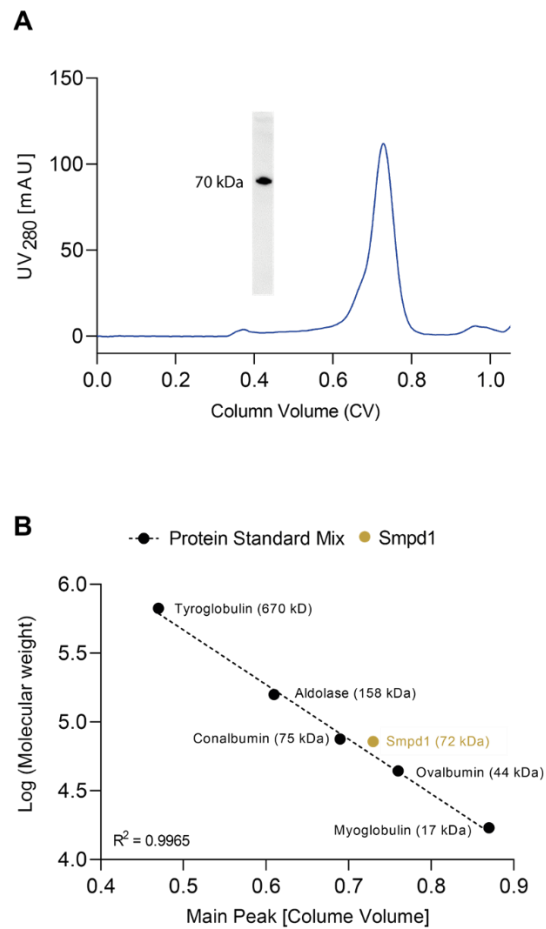


Figure A4: Size exclusion chromatogram of SMPD1 using a Superdex 200 column. A Western Blot against the C-terminal His tag is included, to confirm the presence of the correct protein. B: Comparison of separations of a SEC protein standard mix and intact SMDP1. The protein elutes according to the apparent molecular weight of the monomer (72 kDa) which shows that no aggregate formation has taken place.

Table A1: Calculated values for risk matrix parameters: Expression, occurrence in monoclonal antibody (mAb) products, polysorbate (PS) degradation activity, localization. Rating approach is indicated in brackets in the top of the column. The mean of all values for a hydrolase resulted in the final score.

	Expression (Highest TPM = 10, TPM/highest TPM · 10)	Occurrence in mAb products (in 3 of 7 pro- jects = 10)	PS degradation activity (0% on day 14 = 10;100% = 0)	Localization (100% Extracel- lular = 10)	Final Score (Mean of all pa- rameters)
Ces1	0,11	3,33	3,83	2,84	2,02
Ces1f	3,20	3,33	5,00	7,33	3,77
Ces2c	0,00	3,33	6,34	7,26	3,39
Iah1	0,43	3,33	4,83	0,56	1,83
Lipa	10,00	10,00	4,86	0,89	5,15
Lpla2	0,27	6,67	10,00	5,75	4,54
Paf- ah	1,46	3,33	10,00	9,82	4,92
Ppt1	1,64	10,00	5,38	7,53	4,91

Appendix B Supplementary Material Chapter 4

Supplemental figures present the $\log(\alpha)$ contour plots for CEX and MMC respectively, showing all the mAb formats and HCP impurities that were screened in this study.

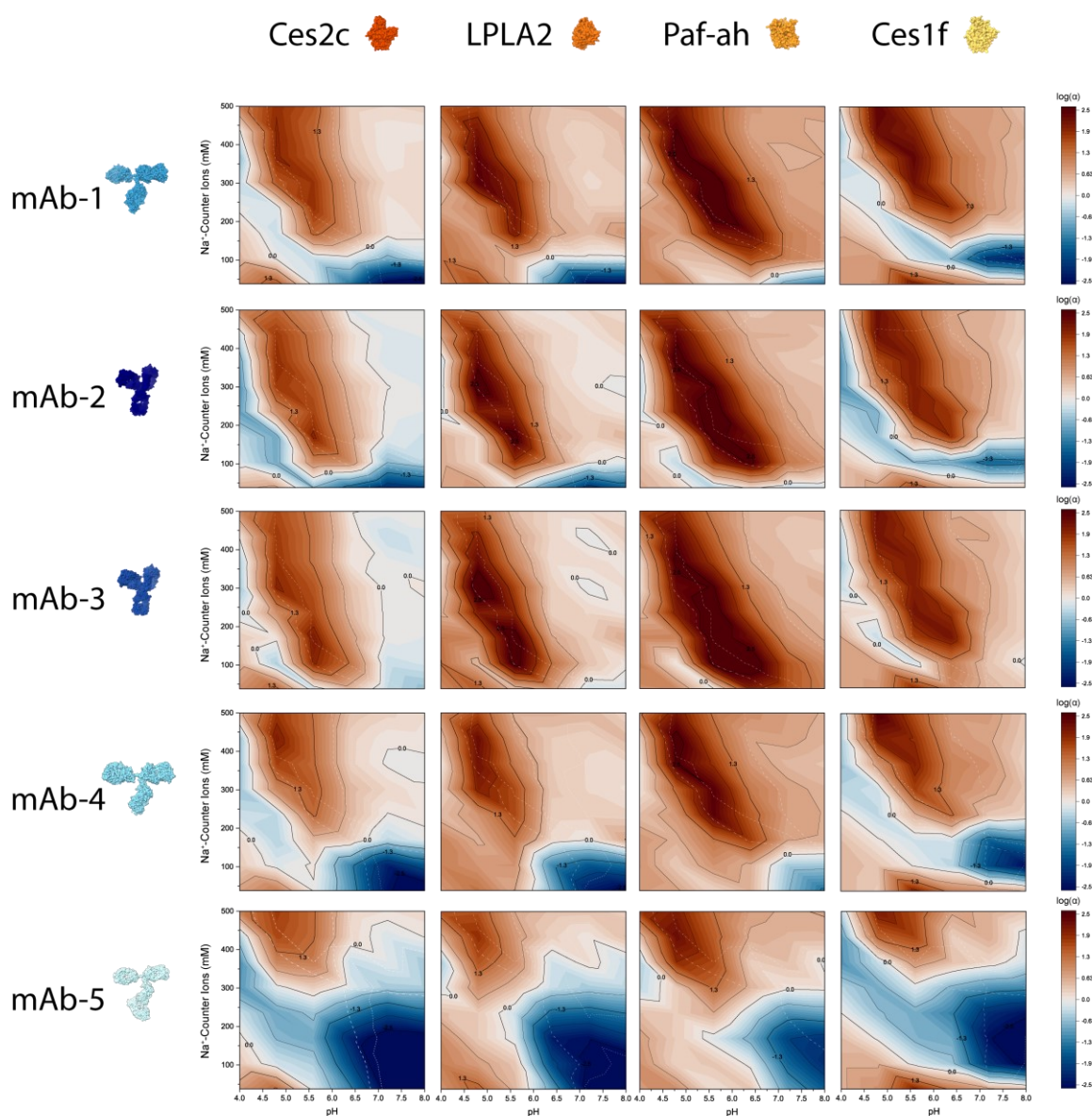


Figure B1: Separation conditions between different mAb formats and polysorbate hydrolases with PorosTM XS resin.

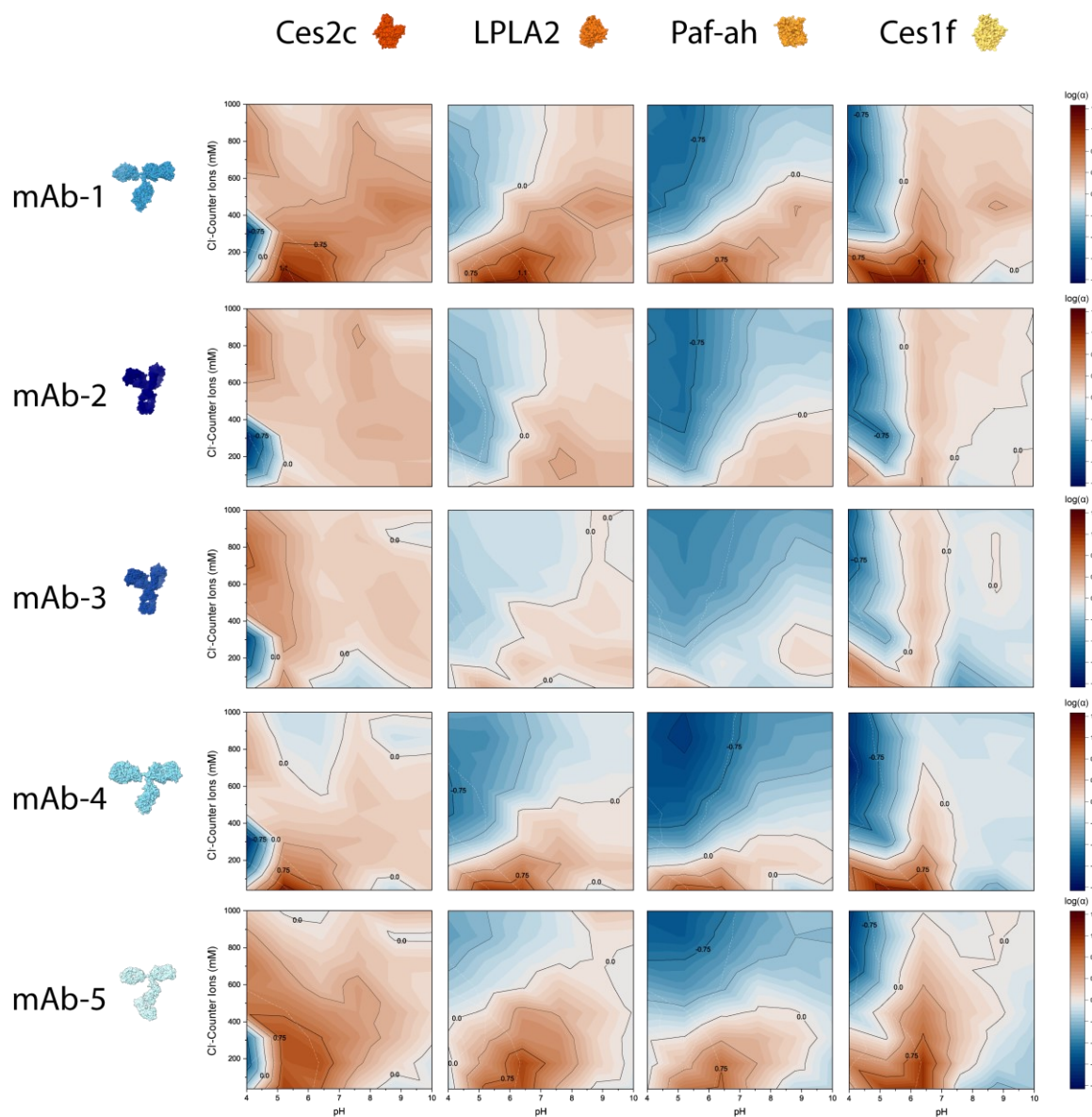


Figure B2: Separation conditions between different mAb formats and polysorbate hydro-lases with Capto adhere resin.

Appendix C Supplementary Material Chapter 5

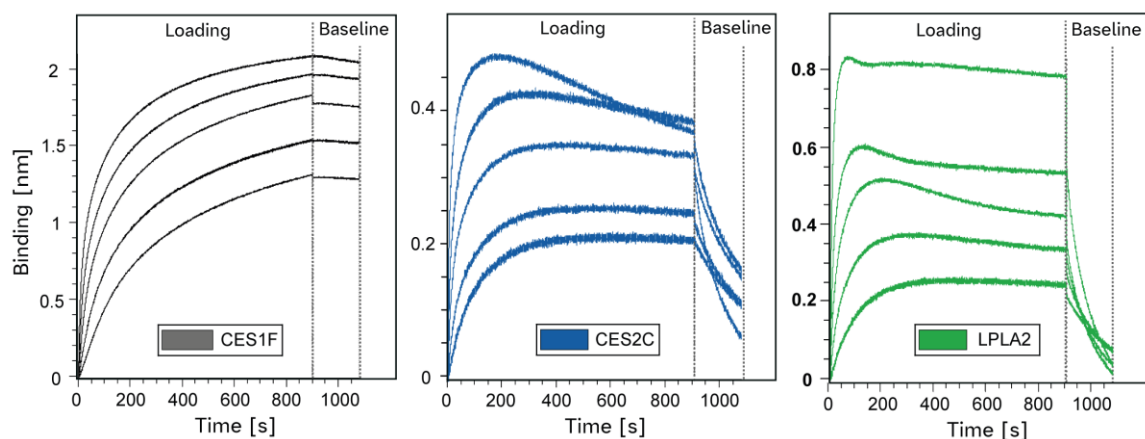


Figure C1: Immobilization profiles of His-tagged recombinant HCPs on HIS2 biosensors. Sensorgrams show the loading and baseline phases for CES1F, CES2C, and LPLA2 captured via their His₆-tags on HIS2 biosensors. Ligand concentrations ranged from 1 to 40 $\mu\text{g/mL}$. The data illustrate the initial association of the HCPs with the biosensor surface, followed by a baseline phase that was not stable across measurements.

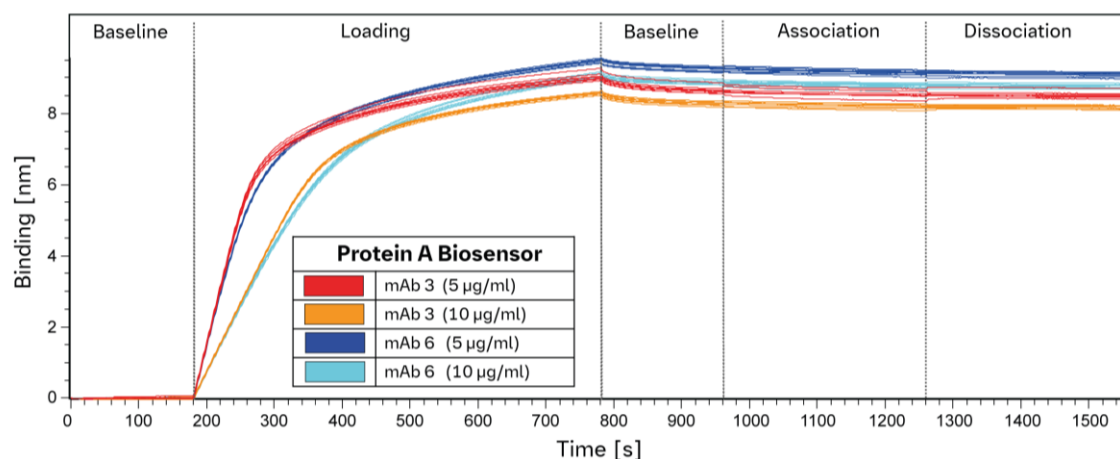


Figure C2: BLI sensorgrams of HCP-mAb interactions using Protein A biosensors. Sensorgrams display the interaction profiles of mAb 3 (IgG4) and mAb 6 (IgG1), immobilized on Protein A biosensors at concentrations of 5 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$, respectively. The analytes CES1F and PAF-AH were applied in a concentration range from 1.5 to 1923 nm. Each trace includes baseline, antibody loading, second baseline, association and dissociation phases. Control measurements on unloaded sensors were included to assess nonspecific binding.

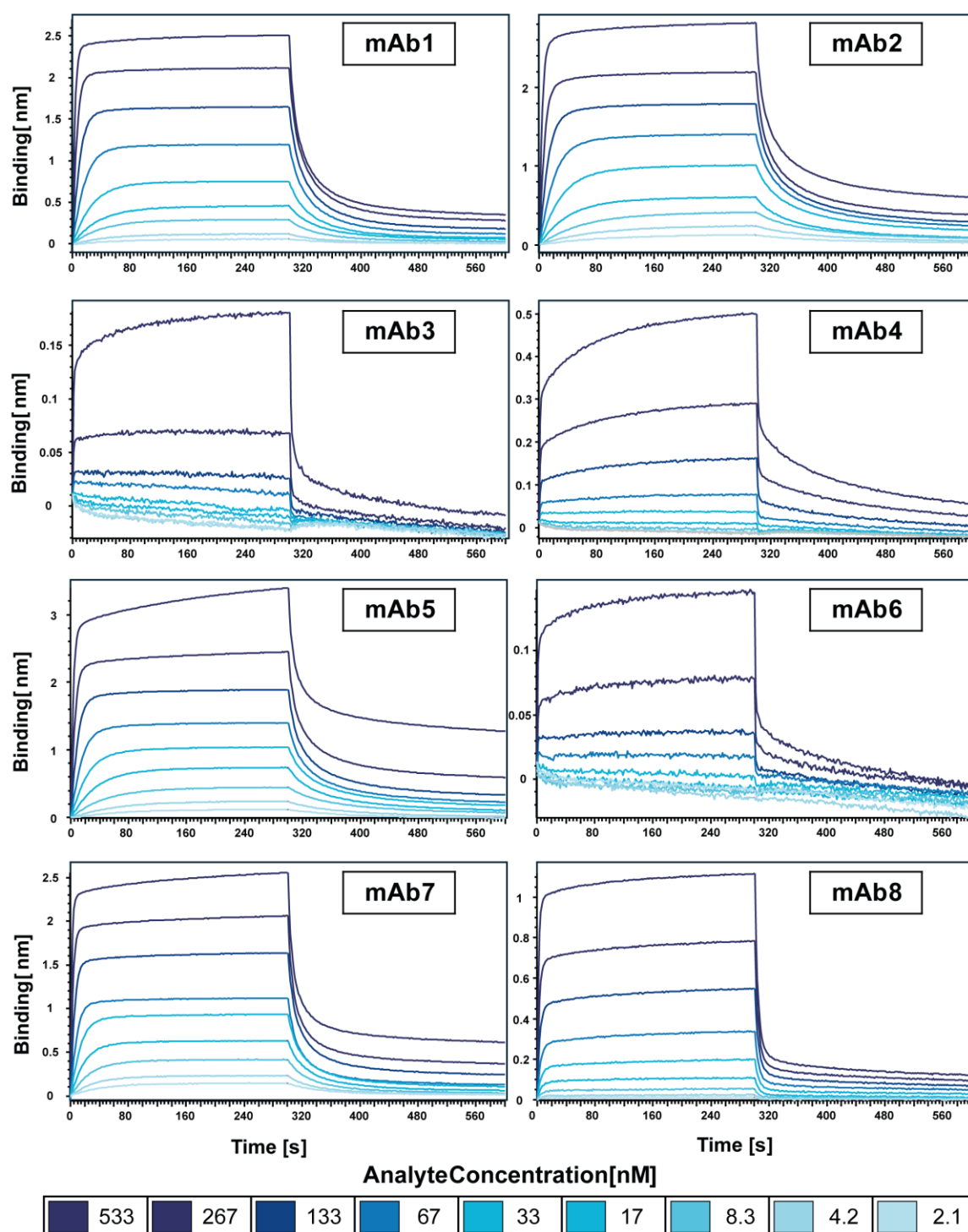


Figure C3: Representative BLI sensorgrams showing the interaction of CES1F with eight monoclonal antibodies. A: mAb 1; B: mAb 2; C: mAb 3; D: mAb 4; E: mAb 5; F: mAb 6; G: mAb 7; H: mAb 8. Antibody concentrations ranged from 2 to 533 nM, as indicated by the color legend. Sensorgrams displayed typical association and dissociation phases. Signal intensity increases with antibody concentration and varies between mAbs, reflecting differences in binding behavior while some show biphasic patterns.

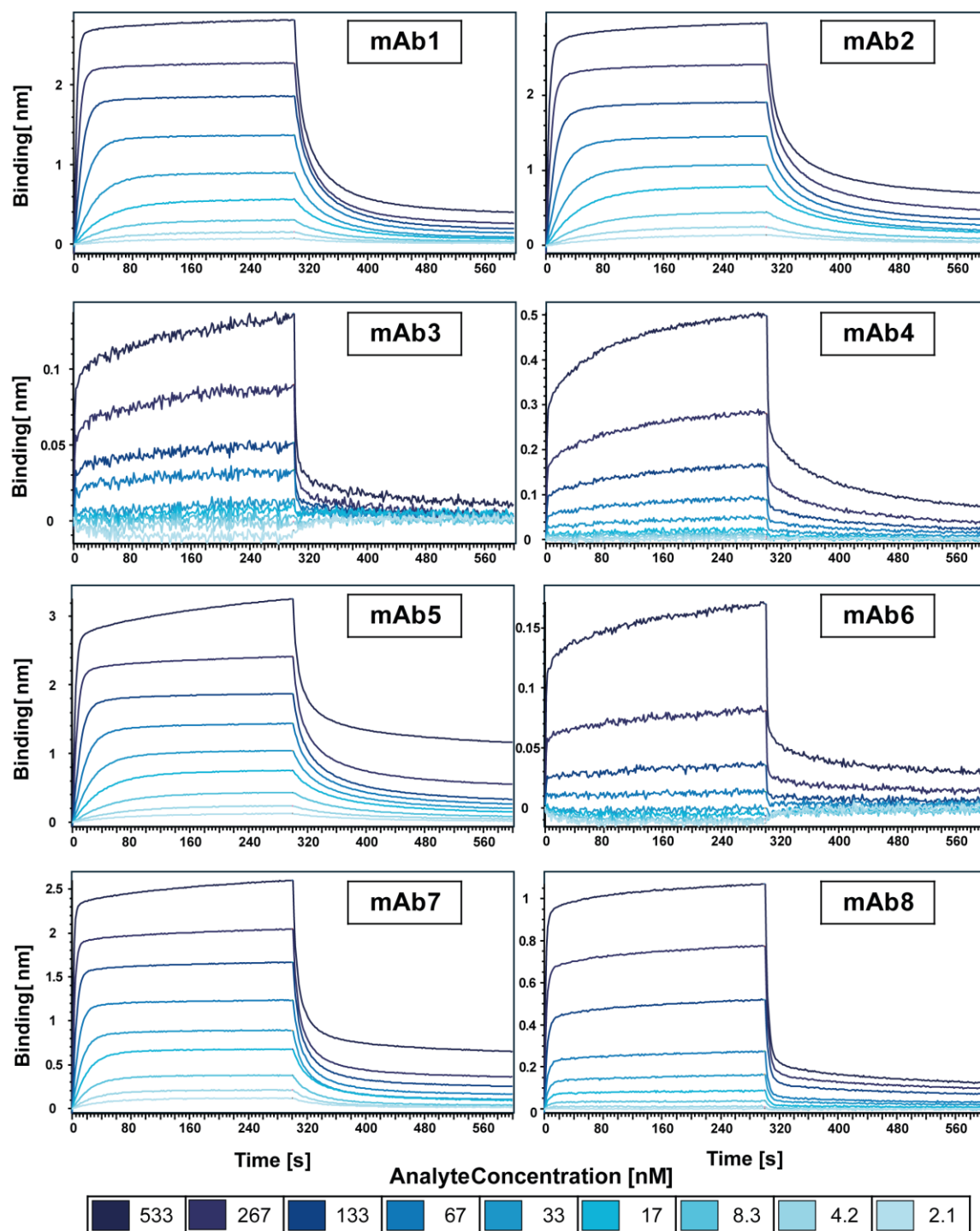


Figure C4: Representative BLI sensorgrams showing the interaction of LPLA2 with eight monoclonal antibodies. A: mAb 1; B: mAb 2; C: mAb 3; D: mAb 4; E: mAb 5; F: mAb 6; G: mAb 7; H: mAb 8. Antibody concentrations ranged from 2 to 533 nM, as indicated by the color legend. Sensorgrams displayed typical association and dissociation phases. Signal intensity increases with antibody concentration and varies between mAbs, reflecting differences in binding behavior while some show biphasic patterns.

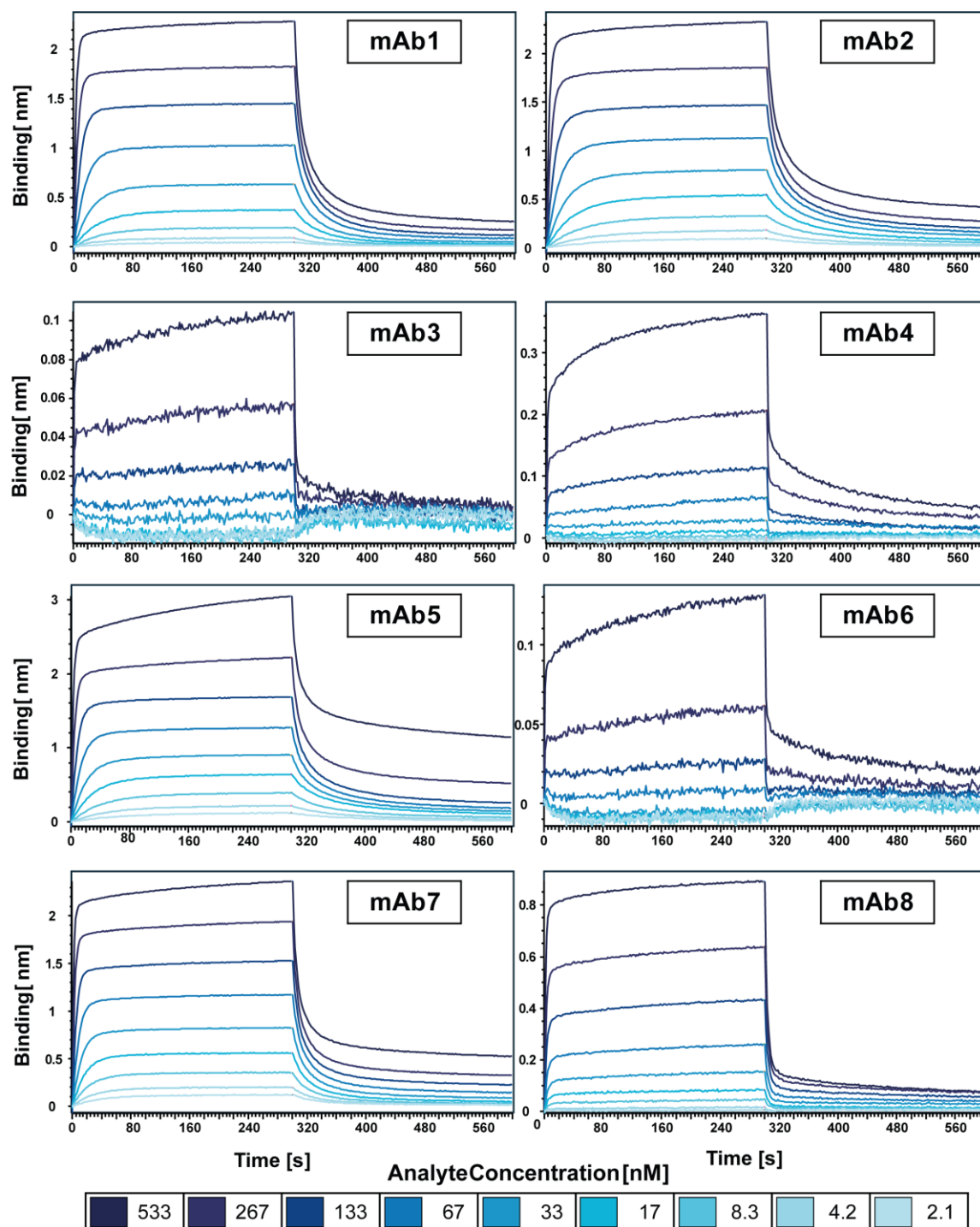


Figure C5: Representative BLI sensorgrams showing the interaction of PAF-AH with eight monoclonal antibodies. A: mAb 1; B: mAb 2; C: mAb 3; D: mAb 4; E: mAb 5; F: mAb 6; G: mAb 7; H: mAb 8. Antibody concentrations ranged from 2 to 533 nM, as indicated by the color legend. Sensorgrams displayed typical association and dissociation phases. Signal intensity increases with antibody concentration and varies between mAbs, reflecting differences in binding behavior while some show biphasic patterns.

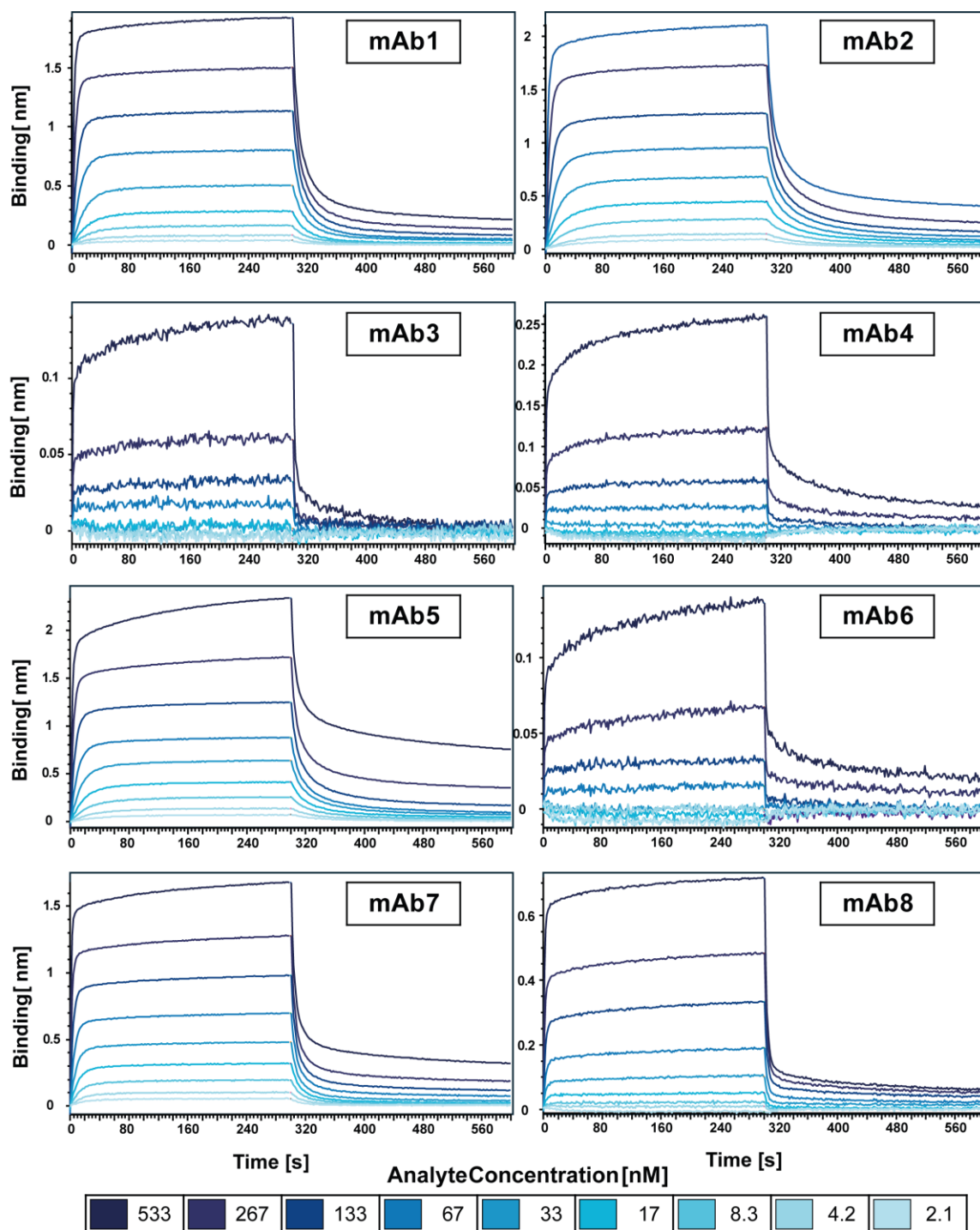


Figure C6: Representative BLI sensorgrams showing the interaction of PPT1 with eight monoclonal antibodies. A: mAb 1; B: mAb 2; C: mAb 3; D: mAb 4; E: mAb 5; F: mAb 6; G: mAb 7; H: mAb 8. Antibody concentrations ranged from 2 to 533 nM, as indicated by the color legend. Sensorgrams displayed typical association and dissociation phases. Signal intensity increases with antibody concentration and varies between mAbs, reflecting differences in binding behavior while some show biphasic patterns.

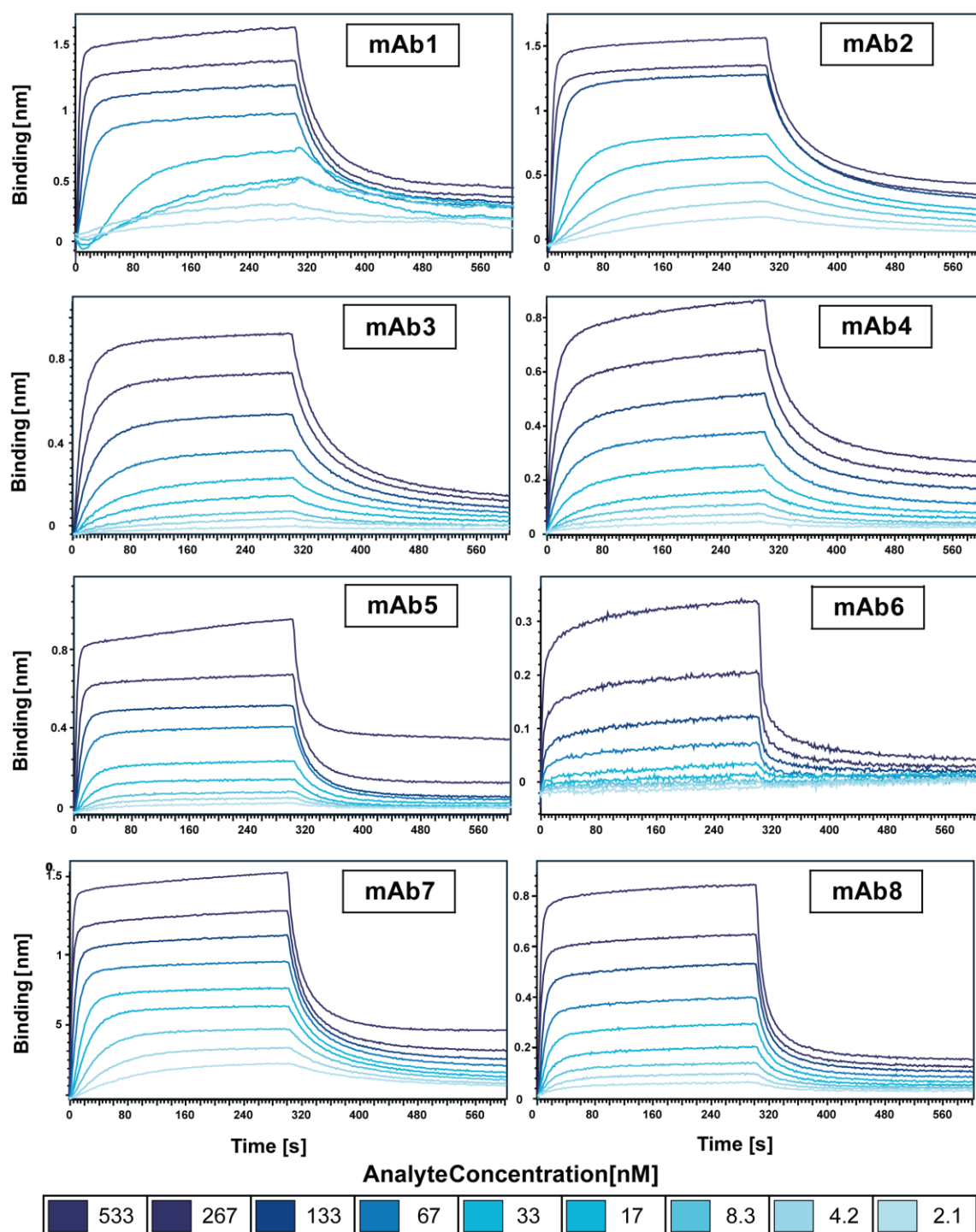


Figure C7: Representative BLI sensorgrams showing the interaction of PLBL2 with eight monoclonal antibodies. A: mAb 1; B: mAb 2; C: mAb 3; D: mAb 4; E: mAb 5; F: mAb 6; G: mAb 7; H: mAb 8. Antibody concentrations ranged from 2 to 533 nM, as indicated by the color legend. Sensorgrams displayed typical association and dissociation phases. Signal intensity increases with antibody concentration and varies between mAbs, reflecting differences in binding behavior while some show biphasic patterns.

Appendix D Supplementary Material Chapter 6

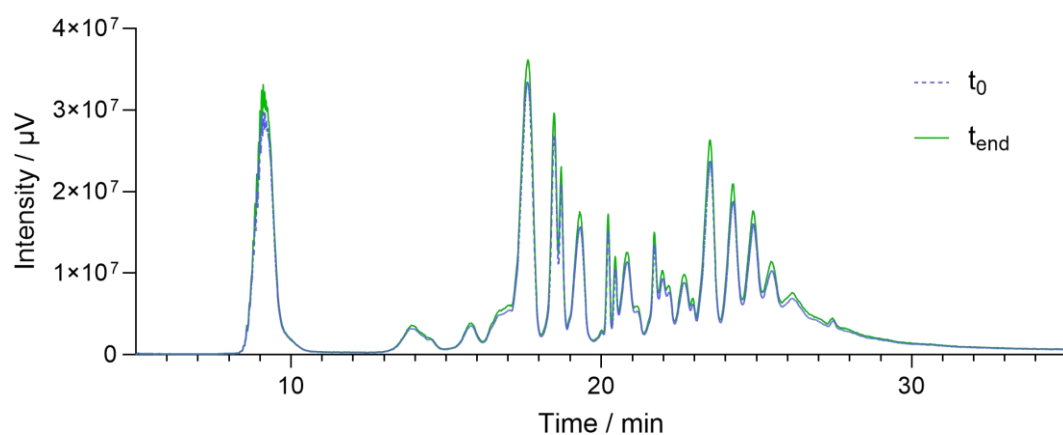


Figure D1: Representative total ion chromatograms of a PS20 blank sample at the start of incubation (t_0) and at the end of the stability study after 60 days (t_{end}). The overlay demonstrates the absence of oxidative degradation, as indicated by the unchanged peak pattern and intensity distribution.